

University of Groningen

Improving the response to molecular targeting of the TRAIL death receptors in colon cancer cells

Pennarun, Bodvaël

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:
2010

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Pennarun, B. (2010). *Improving the response to molecular targeting of the TRAIL death receptors in colon cancer cells*. s.n.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Improving the response to molecular targeting of the TRAIL death receptors in colon cancer cells

Bodvaël Pennarun

Bodvaël Pennarun

Improving the response to molecular targeting of the TRAIL death receptors in colon cancer cells

ISBN: 978-90-367-4437-9 (book)

ISBN: 978-90-367-4438-6 (digital)

© Copyright 2010, Bodvaël Pennarun; Groningen, the Netherlands

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronically, mechanically, by photocopying, recording, or otherwise, without the prior consent of the author.

Cover design: Bodvaël Pennarun

Layout: Bodvaël Pennarun

Printed by Ipskamp drukkers, Enschede, the Netherlands

The publication of this thesis was financially supported by:

University Medical Center Groningen, Novartis Pharma BV, Merck Sharp & Dohme BV,
Stichting Werkgroep Interne Oncologie



**rijksuniversiteit
 groningen**

Improving the response to molecular targeting of the TRAIL death receptors in colon cancer cells

Proefschrift

ter verkrijging van het doctoraat in de
 Medische Wetenschappen
 aan de Rijksuniversiteit Groningen
 op gezag van de
 Rector Magnificus, dr. F. Zwarts,
 in het openbaar te verdedigen op
 maandag 28 juni 2010
 om 13.15 uur

door

Bodvaël Erwan Pennarun

geboren op 14 mei 1978
 te Rennes, Frankrijk

Promotores: Prof. dr. J.H. Kleibeuker
Prof. dr. E.G.E. de Vries

Copromotor: Dr. S. de Jong

Beoordelingscommissie: Prof. dr. H. Moshage
Prof. dr. W.J. Quax
Prof. dr. A.G.J. van der Zee

Contents

Chapter 1	General introduction	7
Chapter 2	Playing the DISC: turning on TRAIL death receptor-mediated apoptosis in cancer <i>BBA-Rev Cancer 2010; 1805: 123-140.</i>	17
Chapter 3	Downregulation of active caspase 8 as a mechanism of acquired TRAIL resistance in mismatch repair proficient colon carcinoma cell lines <i>Submitted for publication</i>	75
Chapter 4	Modulation of TRAIL resistance in colon carcinoma cells: Different contribution of DR4 and DR5 <i>Submitted for publication</i>	101
Chapter 5	Inhibition of tyrosine kinase-dependent PI3K activation sensitizes colon cancer cells specifically to DR5-mediated apoptosis but not to rhTRAIL <i>Submitted for publication</i>	127
Chapter 6	Sorafenib plus aspirin promotes TRAIL-induced apoptosis by targeting FLIP and Mcl-1 and potentiates growth inhibition in colon cancer cells <i>Manuscript in preparation</i>	153
Chapter 7	Summary of the results, general discussion and future perspectives	181
Chapter 8	Summary in Dutch (Nederlandse samenvatting)	197
	Acknowledgements	203

Chapter 1

General introduction

The term 'apoptosis', derived from the Greek *apo* (- from), *ptosis* (- falling), refers, in the medical world, to the programmed death of unwanted cells in an organism (1). Apoptosis is essential for proper development and tissue integrity. Due to the tight regulation of this process, also called programmed cell death (PCD), cells are eliminated without harm to the surrounding tissues. (Epi)genetic alterations can disrupt a cell's ability to enter apoptosis and thereby threaten homeostasis. The uncontrolled proliferation of these damaged cells can cause several diseases, including cancer.

Apoptosis can be induced through two intertwined pathways known as the **extrinsic** and the **intrinsic** pathways, depending on the initiating event (2). Apoptotic cell death via the **intrinsic** (stress) pathway is initiated by cellular changes such as DNA damage (e.g., following radiation or treatment with chemotherapeutic drugs), which causes destabilization of the mitochondrial membrane via the proapoptotic Bcl-2 homologs Bax and Bak. The **extrinsic** pathway is initiated by binding of extracellular death ligands to their cognate death receptors at the cell surface. This extrinsic pathway can also activate the intrinsic pathway of apoptosis via cleavage of the proapoptotic Bcl-2 homology domain 3 (BH3)-only protein Bid. Both pathways strongly depend on sequential activation of a family of proteins called caspases, although the involvement of many non-caspase proteins is also critical.

Chemotherapeutic agents can stimulate the mitochondrial pathway of apoptosis in cancer cells, but their effects are often hampered by the intrinsic resistance of these cells to apoptosis mediated by this pathway. Combining chemotherapeutic agents and stimulation of the extrinsic pathway has emerged as a rational strategy to bypass blockade of the intrinsic apoptotic pathway in cancer cells. In the clinic, tumor necrosis factor-related apoptosis-inducing ligand receptors 1 and 2 (TRAIL-R1 and TRAIL-R2), also named death receptors 4 and 5 (DR4 and DR5), are the most interesting targets for stimulation of the extrinsic pathway. Early clinical studies have indeed shown that TRAIL receptor ligands, such as recombinant human TRAIL (rh)TRAIL or agonistic anti-TRAIL receptor antibodies, are well-tolerated in patients (3).

Colorectal cancer is one of the most frequent forms of malignancy. 150,000 new cases are diagnosed each year in the U.S. alone. Colorectal cancer is also one of the most common causes of cancer-related death in the general population, second to lung cancer (4). The development of colorectal cancer has been associated with lack of exercise, obesity, smoking, alcohol consumption, a low-fibre, high-fat and high red-meat diet.

Although these factors contribute to the development of the disease, notably in its sporadic form, genetic predisposition also plays a major role in a subset of patients. Two main forms of inherited genetic disorders exist, namely familial adenomatous polyposis (FAP) and Lynch syndrome, formerly known as hereditary non-polyposis colorectal cancer (5,6). Although hereditary disorders account for less than 10% of colorectal cancers, their association with a high incidence of these cancers makes them a major concern. Tumor resection constitutes the standard treatment for localized colorectal cancer while adjuvant chemotherapy is used to improve survival in a patients with metastases to the regional lymph nodes, i.e. stage III disease (7). The best curative option for those 20 % of patients who present with metastatic colorectal cancer at time of diagnosis remains surgical removal of both the primary tumor and the metastases, in particular of those in the liver (8). However, most patients will only benefit from palliative systemic treatment options (7). The five-year survival rates for patients with metastatic disease was only 10-30 % in 2005 (9). The recent addition of bevacizumab and cetuximab to standard chemotherapy for treating colorectal cancer has led to a raise of these survival rates over the last years (7). These encouraging results further support the introduction of novel targeted therapies.

Intrinsic resistance against available therapeutics is frequently observed while acquired resistance can develop during treatment. *In vitro* studies suggest that, despite their resistance to chemotherapeutic drugs, a significant proportion of colon cancer cell lines exhibit sensitivity to extrinsic cell death as induced by rhTRAIL (10). Sensitivity to rhTRAIL and other TRAIL receptor agonists can be further improved by combining these agents with conventional cytotoxic chemotherapies, both *in vitro* in cancer cell lines and *ex vivo* in primary tumor material (11-13). Similar results have been found *in vivo* using human subcutaneous and orthotopic tumor xenograft mouse models (14,15). Early results from phase I and II studies have suggested that the optimal combinations incorporating TRAIL receptor-targeting agents remain to be defined. The aim of this thesis is, therefore, to characterize new combinations that are favorable for inducing TRAIL death receptor-mediated apoptosis in colon cancer cells. We also aimed to define some of the key factors that influence TRAIL sensitivity in colorectal cancer cells, with the prospect of translating these findings into potential therapeutic strategies.

Many studies have shown that by targeting the extrinsic apoptosis pathway, TRAIL can increase the effects of conventional anti-cancer treatments on the intrinsic pathway. In **chapter 2**, we present an extensive overview of the processes regulating the initial step of TRAIL death receptor-mediated apoptosis, the formation of the so-called death-inducing signalling complex (DISC). Reaching optimal DISC activity means fully exploiting mitochondria-independent apoptotic pathways while enhancing, via cleavage of the BH3-only protein Bid, apoptotic stress on the mitochondria as induced by conventional treatment modalities.

Although colon cancer cells are more sensitive to TRAIL than their healthy counterpart, some cancer cells are either only moderately sensitive or completely resistant to TRAIL (10). Identifying the critical steps regulating TRAIL sensitivity will help to define possible targets for potentiating TRAIL-induced apoptosis. The potential for cancer cells to gradually acquire TRAIL resistance over time has been used previously to establish *in vitro* models of TRAIL resistance and to study the cellular changes associated with loss of TRAIL sensitivity. Previous studies have identified Bax as a key protein lost during drug resistance acquisition in colon cancer cells, including resistance to TRAIL (16,17). These findings were obtained in mismatch repair (MMR)-deficient cells, which account for less than 20% of all colorectal cancers and are predisposed to mutations in genes with short tandem repeat sequences such as Bax (6,16). In **chapter 3**, we establish a model of TRAIL resistance in MMR-proficient colon cancer cells to identify the key protein(s), the expression of which may be preferentially altered during the acquisition of TRAIL resistance. Cellular differences between the TRAIL-sensitive SW948 cell line and its TRAIL-resistant daughter cell line SW948-TR are studied using a combination of survival assays, apoptosis quantification with acridine orange staining and Western blotting. The functional significance of the ratio between levels of caspase 8 and its competitive inhibitor FLIP for TRAIL sensitivity is evaluated using short interfering RNA. We also make use of several compounds which effectively re-establish a pro-apoptotic (high) caspase 8/FLIP ratio in SW948-TR cells.

In addition to rhTRAIL, TRAIL-R1- and TRAIL-R2-specific antibodies can also be used to stimulate TRAIL death receptor-mediated apoptosis (3). Although these agents all stimulate the TRAIL apoptotic pathway, their efficacy might differ due to their ability to stimulate, respectively, TRAIL-R1/-R2, TRAIL-R1 or TRAIL-R2. In **chapter 4**, we compare the potency of these various ligands for inducing apoptosis in SW948 and

SW948-TR cells *in vitro*. Searching for optimal combinations, we also compare the efficacy of the cytokine interferon (IFN)- γ and of the proteasome inhibitor MG-132 in modulating sensitivity to the various TRAIL receptor ligands in our cell line model. In addition, the differential ability of TRAIL-R1 and TRAIL-R2 transduce apoptosis is investigated.

Cetuximab, a humanized anti-epidermal growth factor receptor (EGFR) antibody, has recently been added to the arsenal of therapies used against colorectal cancer in the clinic (7). This reflects the current shift toward the development of specific anti-cancer therapies targeting key survival pathways. The insulin-like growth factor 1 receptor (IGF-1R), which is often over-activated in cancer, is now gaining much attention (18). We hypothesize that inhibition of IGF-1R in colon cancer cells might alter the balance between survival- and apoptosis-related factors, thereby increasing sensitivity to TRAIL. In **chapter 5**, we test the effects of the small molecule IGF-1R inhibitor NVP-AEW541 on cell survival in our TRAIL resistant colon cancer cell model. In view of the discrepancies seen between the effects of the various TRAIL receptor agonists in combination with IFN- γ and MG-132, we also compare the effects of rhTRAIL and the anti-TRAIL death receptor antibodies following IGF-1R inhibition. The contribution of the downstream IGF-1R substrate phosphatidylinositol 3-phosphate (PI3K) is also studied and experiments were expanded to another colon cancer cell line, namely Colo205.

Although the precise mode of action is still debated, epidemiological studies have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) have chemopreventive effects *in vivo*, notably in colorectal cells (19). Although NSAIDs can interfere with pathways that play an important role in cancer, the NSAID celecoxib failed to show anti-cancer properties in phase II studies (20,21). *In vitro* and human tumor xenograft mouse studies demonstrated that aspirin used in combination with rhTRAIL has anti-cancer activity (22,23). The obstacle constituted by the high concentrations of aspirin required could be potentially overcome through combinatorial approaches that would enhance the efficacy of aspirin and allow lowering the needed dose. Sorafenib is a broad spectrum kinase inhibitor targeting the RAF/MEK/ERK pathway and several tyrosine kinase receptors associated with angiogenesis and tumorigenesis (24). This drug has been approved for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma, and its potential use against a number of other malignancies is under evaluation in over 380 clinical studies (Source:

ClinicalTrials.gov, March 2010). Because aspirin and sorafenib have similar cellular targets, such as the cell cycle protein cyclin D1 (25,26) and the anti-apoptotic protein Mcl-1 (27,28), we hypothesize that these two drugs might be able to interact with each other. In **chapter 6**, we combine sorafenib and aspirin in a panel of colon cancer cell lines and investigate the effects on cell survival. Furthermore, sorafenib and aspirin have independently demonstrated very attractive sensitizing properties for TRAIL receptor-mediated apoptosis *in vitro*, albeit at doses bordering the clinically achievable range (19,29). We combine physiologically sustainable doses of both aspirin and sorafenib with TRAIL to investigate potential cross-amplification of their pro-apoptotic effects *in vitro*. The combination of aspirin and sorafenib led to downregulation of at least two anti-apoptotic proteins in several cell lines. The possible contribution of these proteins to TRAIL resistance is investigated. Finally, we assess the physiological relevance of the lesser-known of our two targets by evaluating its basic expression in normal colorectal epithelium, colorectal adenoma and colorectal carcinoma using immunohistochemical staining of patient tissue. Material from patients with the sporadic form of the disease and with Lynch syndrome has been included in this study.

Finally, **chapter 7** summarizes the results of this thesis and proposes future research lines for the TRAIL-based treatment of colon cancer, motivated by the work presented herein.

References

1. Kerr JF, Wyllie AH and Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; 26: 239-257.
2. Hengartner M. The biochemistry of apoptosis. *Nature* 2000; 407: 770-776.
3. Oldenhuis CNAM, Stegehuis JH, Walenkamp AME, de Jong S and de Vries EGE. Targeting TRAIL death receptors. *Curr Opin Pharmacol* 2008; 8: 433-439.
4. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T and Thun M. Cancer Statistics, 2008. *CA Cancer J Clin* 2008; 58: 71-96.
5. Midgley R and Kerr D. Colorectal cancer. *The Lancet* 1999; 353: 391-399.
6. Peltomaki P. Deficient DNA mismatch repair: a common etiologic factor for colon cancer. *Hum Mol Genet* 2001; 10: 735-740.
7. Segal N and Saltz L. Evolving Treatment of Advanced Colon Cancer. *Annu Rev Med* 2009; 60: 207-219.
8. Fong Y, Cohen AM, Fortner JG, Enker WE, Turnbull AD, Coit DG, Marrero AM, Prasad M, Blumgart LH and Brennan MF. Liver resection for colorectal metastases. *J Clin Oncol* 1997; 15: 938-946.
9. Kelly H and Goldberg RM. Systemic therapy for metastatic colorectal cancer: current options, current evidence. *J Clin Oncol* 2005; 23: 4553-4560.
10. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokhi Z and Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999; 104: 155-162.
11. van Geelen CMM, de Vries EGE and de Jong S. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist Updat* 2004; 7: 345-358.
12. Estes J, Oliver P, Straughn J, Zhou T, Wang W, Grizzle W, Alvarez R, Stockard C, LoBuglio A and Buchsbaum D. Efficacy of anti-death receptor 5 (DR5) antibody (TRA-8) against primary human ovarian carcinoma using a novel ex vivo tissue slice model. *Gynecol Oncol* 2007; 105: 291-298.
13. Koschny R, Holland H, Sykora J, Haas TL, Sprick MR, Ganten TM, Krupp W, Bauer M, Ahnert P, Meixensberger J and Walczak H. Bortezomib sensitizes primary human astrocytoma cells of WHO grades I to IV for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Clin Cancer Res* 2007; 13: 3403-3412.
14. Johnstone RW, Frew AJ and Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer* 2008; 8: 782-798.
15. Zinonos I, Labrinidis A, Lee M, Liapis V, Hay S, Ponomarev V, Diamond P, Zannettino A, Findlay D and Evdokiou A. Apomab, a fully human agonistic antibody to DR5, exhibits potent antitumor activity against primary and metastatic breast cancer. *Mol Cancer Ther* 2009; 8: 2969-2980.

16. Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC and Perucho M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997; 275: 967-969.
17. LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D and Ashkenazi A. Tumor-cell resistance to death receptor--induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nat Med* 2002; 8: 274-281.
18. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008; 8: 915-928.
19. Jalving M, Koornstra JJ, de Jong S, de Vries EGE and Kleibeuker JH. Review article: the potential of combinational regimen with non-steroidal anti-inflammatory drugs in the chemoprevention of colorectal cancer. *Aliment Pharmacol Ther* 2005; 21: 321-339.
20. El Rayes BF, Zalupski MM, Manza SG, Rusin B, Ferris AM, Vaishampayan U, Heilbrun LK, Venkatramanamoorthy R, Shields AF and Philip PA. Phase-II study of dose attenuated schedule of irinotecan, capecitabine, and celecoxib in advanced colorectal cancer. *Cancer Chemother Pharmacol* 2008; 61: 283-289.
21. Maiello E, Giuliani F, Gebbia V, Di Renzo N, Pezzella G, Romito S, Mallamaci R, Lopez M and Colucci G. FOLFIRI with or without celecoxib in advanced colorectal cancer: a randomized phase II study of the Gruppo Oncologico dell'Italia Meridionale (GOIM). *Ann Oncol* 2006; 17 Suppl 7: vii55-vii59.
22. Kim Y, Suh N, Sporn M and Reed J. An Inducible Pathway for Degradation of FLIP Protein Sensitizes Tumor Cells to TRAIL-induced Apoptosis. *J Biol Chem* 2002; 277: 22320-22329.
23. Lu M, Strohecker A, Chen F, Kwan T, Bosman J, Jordan VC and Cryns VL. Aspirin sensitizes cancer cells to TRAIL-induced apoptosis by reducing survivin levels. *Clin Cancer Res* 2008; 14: 3168-3176.
24. Wilhelm S, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujath J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnane L, Lynch M, Auclair D, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post L, Bollag G and Trail P. BAY 43-9006 Exhibits Broad Spectrum Oral Antitumor Activity and Targets the RAF/MEK/ERK Pathway and Receptor Tyrosine Kinases Involved in Tumor Progression and Angiogenesis. *Cancer Res* 2004; 64: 7099-7109.
25. Perugini RA, McDade TP, Vittimberga FJ, Jr., Duffy AJ and Callery MP. Sodium salicylate inhibits proliferation and induces G1 cell cycle arrest in human pancreatic cancer cell lines. *J Gastrointest Surg* 2000; 4: 24-32.
26. Plastaras JP, Kim SH, Liu YY, Dicker DT, Dorsey JF, McDonough J, Cerniglia G, Rajendran RR, Gupta A, Rustgi AK, Diehl JA, Smith CD, Flaherty KT and El Deiry WS. Cell cycle dependent and schedule-dependent antitumor effects of sorafenib combined with radiation. *Cancer Res* 2007; 67: 9443-9454.
27. Iglesias-Serret D, Pique M, Gil J, Pons G and Lopez JM. Transcriptional and translational control of Mcl-1 during apoptosis. *Arch Biochem Biophys* 2003; 417: 141-152.
28. Yu C, Bruzek LM, Meng XW, Gores GJ, Carter CA, Kaufmann SH and Adjei AA. The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. *Oncogene* 2005; 24: 6861-6869.

29. Ricci MS, Kim SH, Ogi K, Plataras JP, Ling J, Wang W, Jin Z, Liu YY, Dicker DT, Chiao PJ, Flaherty KT, Smith CD and El-Deiry WS. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* 2007; 12: 66-80.

Playing the DISC: Turning on TRAIL death receptor-mediated apoptosis in cancer

Bodvaël Pennarun^{1,2#}

Annemieke Meijer^{1#}

Elisabeth G.E. de Vries¹

Jan H. Kleibeuker²

Frank Kruyt¹

Steven de Jong¹

both authors contributed equally

Departments of ¹Medical Oncology and ²Gastroenterology,
University Medical Center Groningen,
University of Groningen, the Netherlands

BBA-Rev Cancer 2010; 1805: 123-140.

Abstract

Formation of the pro-apoptotic death-inducing signaling complex (DISC) can be initiated in cancer cells via binding of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to its two proapoptotic receptors, TRAIL receptor 1 (TRAIL-R1) and TRAIL-R2. Primary components of the DISC are trimerized TRAIL-R1/-R2, FADD, caspase 8 and caspase 10. The anti-apoptotic protein FLIP can also be recruited to the DISC to replace caspase 8 and form an inactive complex. Caspase 8/10 processing at the DISC triggers the caspase cascade, which eventually leads to apoptotic cell death. Besides TRAIL, TRAIL-R1- or TRAIL-R2- selective variants of TRAIL and agonistic antibodies have been designed. These ligands are of interest as anti-cancer agents since they selectively kill tumor cells. To increase tumor sensitivity to TRAIL death receptor-mediated apoptosis and to overcome drug resistance, TRAIL receptor ligands have already been combined with various therapies in preclinical models. In this review, we discuss factors influencing the initial steps of TRAIL apoptosis signaling pathway, focusing on mechanisms modulating DISC assembly and caspase activation at the DISC. These insights will direct rational design of drug combinations with TRAIL receptor ligands to maximize DISC signaling.

Contents

- A. Introduction
- B. TRAIL
- C. TRAIL-induced DISC formation and apoptotic signaling pathway
- D. Combination therapy enhances TRAIL-induced apoptosis
- E. TRAIL combination therapy enhances DISC formation
- F. Loss of TRAIL-R1 and TRAIL-R2 expression
- G. DISC component polymorphisms and functionality
- H. Death receptor-selective TRAIL variants
- I. TRAIL death receptor-specific antibodies
- J. Regulation of TRAIL death receptor-mediated apoptotic signaling at the cell membrane
 - 1. *TRAIL-R1 and TRAIL-R2 aggregation promotes DISC formation*
 - 2. *Lipid raft localization stimulates TRAIL death receptor aggregation*
 - 3. *Receptor internalization hampers TRAIL death receptor-mediated apoptosis*
- K. Post-translational modifications of the DISC components
 - 1. *TRAIL death receptor O-glycosylation facilitates receptor aggregation*
 - 2. *TRAIL-R1 S-nitrosylation stimulates caspase 8 cleavage*
 - 3. *S-palmitoylation of TRAIL-R1 promotes its oligomerization*
 - 4. *Caspase 8 phosphorylation prevents its cleavage at the DISC*
 - 5. *FLIP phosphorylation lowers its affinity for the DISC*
 - 6. *DISC protein ubiquitination differentially regulates DISC composition*
- L. Influence of survival pathways on DISC formation
 - 1. *The pro- and anti-DISC effects of protein kinase C*
 - 2. *The ERK1/2 signaling pathway inhibits caspase 8 cleavage*
 - 3. *The anti-DISC effects of active CKI α and CKII α*
- M. DISC regulation by recruitment of additional proteins
 - 1. *PED/PEA-15 blocks caspase 8 cleavage at the DISC*
 - 2. *Blockade of DISC formation by the DDX3/GSK3/cIAP-1 capping complex*
 - 3. *The pro- and anti-DISC properties of the various IG20 gene family isoforms*
 - 4. *Regulation of cell membrane TRAIL-R1 levels by the signal recognition particle complex*
- N. Conclusions

A. Introduction

Programmed apoptotic cell death (or apoptosis) is a process by which unwanted cells are eliminated from multicellular organisms in a non-harmful way (1-4). The list of such unwanted cells includes malignant cells, infected cells or certain lymphocytes. Interestingly, although cancer originates from uncontrolled cell proliferation, cancer cells tend to exhibit more apoptosis than normal cells (5-7). This property may contribute to the effectiveness of some of the cytotoxic and targeted agents used in the clinic (8).

Apoptosis can be initiated via two canonical pathways: the intrinsic and the extrinsic pathway, which both ultimately activate the same effector caspases and apoptosis effector molecules. Extrinsic cell death is triggered by the binding of tumor necrosis factor (TNF) superfamily ligands to their cognate receptors, stimulating their respective apoptotic pathways. Although the CD95-mediated apoptotic pathway is by far the best understood of these pathways, the TRAIL death receptor-mediated apoptotic pathway is clinically more attractive while it still shares numerous key components with the CD95-mediated apoptotic pathway (9,10). The trimeric β -sheet endogenous protein TRAIL binds to its trimeric membrane TRAIL-R1 and TRAIL-R2, initiating the formation of death-inducing signaling complexes (DISCs) that stimulate the caspase cascade, eventually leading to apoptosis. Although CD95-mediated apoptosis has offered a very useful mechanistic model for the TRAIL apoptotic pathway, sensitivity to stimulation of these two pathways is not necessarily the same in identical cancer cells, suggesting critical regulatory differences (11,12). Importantly, the clinical development of TRAIL receptor ligands is markedly more advanced than that of CD95 ligands because of the minimal toxicity of the former and the high toxicity of the latter observed in preclinical studies (13,14).

Two types of cancer cells have been identified based on their sensitivity to stimulation of the extrinsic pathway. Type I cells do not need the mitochondria to enter apoptosis, while type II cells require mitochondrial amplification of the extrinsic signal to achieve sufficient caspase cleavage for the initiation of apoptosis (15). Since most cancer cells express membrane TRAIL death receptors, there is a strong rationale for modulating TRAIL-induced apoptosis directly at the DISC or at the level of the intrinsic pathway. Many of the drugs that have been combined with TRAIL receptor ligands thus

far have been chosen based on their primary ability to activate the mitochondrial pathway of apoptosis; the goal was thereby to stimulate both the intrinsic and extrinsic pathway. Substantial data show that TRAIL death receptor-mediated apoptosis can already be modulated at the cell membrane itself. This review will discuss the proteins and pathways that offer interesting or novel targets for combination with TRAIL receptor-targeted therapies, with a focus on approaches influencing caspase 8 activation at the DISC. We will emphasize on TRAIL receptor ligand-specific and drug-specific abilities to promote DISC formation, TRAIL death receptor signaling modulation in close proximity to the cell membrane and regulation of DISC formation at the intracellular level.

B. TRAIL

TRAIL was first identified independently by Wiley *et al.* and Pitti *et al.* (10,16). This naturally-occurring ligand is, in its recombinant human (rh) form rhTRAIL, a promising therapeutic agent. This is in part due to TRAIL's ability to induce apoptosis in a wide variety of tumor cells while sparing normal cells. Endogenous TRAIL is expressed on natural killer (NK) cells, macrophages, T-cells, and dendritic cells and is thought to function as an immune defense mechanism by killing virus-infected and malignant cells (17). TRAIL knockout (-/-) mice were viable and did not display any hematological defects (18). An important role for TRAIL in tumor immune surveillance was demonstrated using TRAIL-/- mice. Compared to wild type (wt) mice, the TRAIL-/- mice showed increased susceptibility to experimental as well as spontaneous tumor metastases and showed increased tumor initiation following treatment with the chemical carcinogen 3-methylcholanthrene (MCA) (18). In addition, administration of a neutralizing monoclonal antibody against endogenous TRAIL increased MCA-induced tumor development in wt mice (19). In TRAIL receptor-deficient mice, the primary tumor as induced by the carcinogen 7,12-dimethyl-benz-anthracene (DMBA) and the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) were unaffected by TRAIL neutralization (20). However, TRAIL receptor-deficient mice showed enhanced lymph node metastases, suggesting a more specific role for the TRAIL-TRAIL receptor interaction in the prevention of metastases.

Several TRAIL molecules have been generated to utilize TRAIL as an anti-cancer agent, namely histidine (his)-tagged TRAIL, leucine zipper (LZ)-TRAIL, isoleucine zipper (IZ)-TRAIL, flag-tagged TRAIL and recombinant human (rh)TRAIL. A central zinc atom bound to cysteine-230 of each rhTRAIL subunit is essential for stability and function of the trimer rhTRAIL molecule (21). LZ-TRAIL was formed by linkage of exogenous leucine zipper to the extracellular region of TRAIL, resulting in a stable trimeric molecule. His-tagged TRAIL and flag-tagged TRAIL induced apoptosis in cultured human hepatocytes and in human brain slices respectively, raising concerns about toxicity (22,23). *In vitro* and *in vivo* experiments with LZ-TRAIL and rhTRAIL demonstrated that these molecules, as stable trimers devoid of a his- or flag-tag, can be used safely without causing (liver) toxicity (24-26).

The safety and pharmacokinetics of rhTRAIL as a single agent has been tested in a phase 1 clinical trial. RhTRAIL can be administered safely as drug-related toxicity was mild to moderate and the maximum tolerated dose was not reached. From the 51 patients included in the trial, 1 had a partial response and 17 patients experienced a stable disease (27).

C. TRAIL-induced DISC formation and apoptotic signaling pathway

TRAIL can bind five receptors, including TRAIL-R1/death receptor 4 (DR4) and TRAIL-R2/DR5, which contain a death domain (DD), and two decoy receptors (DcRs), TRAIL-R3/DcR1 and TRAIL-R4/DcR2, without a functional DD. Osteoprotegerin (OPG) is a secreted soluble TRAIL receptor. Two isoforms of TRAIL-R2 have been described, designated TRAIL-R2(L)/DR5L for the longer form and TRAIL-R2(S)/DR5S for the shorter form (28). Both forms are expressed in normal and tumor tissues, but the ratio varied among the tissues and cell lines that were examined. Whether the two isoforms differ functionally is still unclear (28). A decoy function for TRAIL-R3 and TRAIL-R4 has only been demonstrated in overexpression studies (29,30). It has been shown that TRAIL decoy receptors could prevent apoptosis signaling via complex formation with TRAIL-R1 and/or TRAIL-R2, resulting in an ineffective DISC (31,32). Upon binding of TRAIL to the TRAIL death receptors, receptor trimerization occurs, which leads to clustering of their DDs and formation of the DISC comprising of Fas-associated death domain (FADD), caspase 8 and 10 (33). The anti-apoptotic protein FLIP can also be

recruited to the DISC to replace caspase 8 and form an inactive complex (34). While caspase 8 is essential, caspase 10 is not required for apoptosis induction by TRAIL (35). DISC formation results in caspase 8 activation. In so-called type I cells, the extrinsic (or death receptor) pathway is induced whereby active caspase 8 directly activates the effector caspases 3, 6 and 7, eventually triggering apoptosis. In type II cells, the intrinsic (or mitochondrial) pathway is necessary to fully activate caspase 3 via caspase 8-dependent cleavage of the Bcl-2 family member Bid into its active form tBid. Translocation of tBid to the mitochondria induces Bax and Bak to form pores in the outer mitochondrial membrane. This allows release of the pro-apoptotic intermembrane space of mitochondria proteins cytochrome c, apoptosis inducing factor (AIF) and Smac/DIABLO into the cytoplasm. In the presence of dATP, cytochrome c induces the interaction of apoptotic protease activating factor 1 (Apaf-1) with caspase 9 resulting in the formation of the so-called apoptosome in which caspase 9 is activated. This subsequently leads to the activation of effector caspases and apoptosis. In type I cells, this mitochondrial pathway can be activated as well, but is not essential for apoptosis induction.

In addition to activation of apoptosis signaling pathways, TRAIL has been linked to non-apoptotic pathways as well, namely nuclear factor (NF)-kappaB, protein kinase B (PKB/Akt) and the mitogen-activated protein kinase (MAPK) signaling pathways, thereby promoting cell survival. This suggests that there is an important balance between cell death and survival signaling upon TRAIL binding, as extensively reviewed by Falschlehner *et al.* (36).

D. Combination therapy enhances TRAIL-induced apoptosis

TRAIL targets the extrinsic apoptosis pathway, while chemo- and radio-therapy mainly inhibit cell growth and/or activate the intrinsic apoptosis pathway. Combination of either chemo- or radio-therapy with TRAIL can activate both apoptosis pathways and increase their anti-tumor effect. Moreover, activation of both the extrinsic and the intrinsic pathway can initiate crosstalk between the two pathways. Such crosstalk can take place via caspase 8-induced tBid resulting in stronger activation of the mitochondrial apoptotic pathway, or via chemo- or radio-therapy induced enhanced expression levels of TRAIL death receptors.

Combinatorial treatments with TRAIL can therefore overcome tumor cell resistance to single agent therapies. In Table 1, examples are given of combination studies of TRAIL with various therapies such as proteasome inhibitors, DNA damaging agents, non-steroidal anti-inflammatory drugs, and ionizing radiation (37). Combined treatment with these therapies induced the expression of TRAIL-R1 and/or TRAIL-R2 and promoted TRAIL-induced apoptosis. In many studies, the enhancement of TRAIL-induced apoptosis has only been attributed to TRAIL death receptor upregulation. The overview presented in Table 1 demonstrates that there is a large variation in drug- or irradiation-induced TRAIL-R1 and/or TRAIL-R2 upregulation. Although TRAIL-R2 was upregulated more frequently than TRAIL-R1, there is no drug that specifically upregulates either TRAIL-R1 or TRAIL-R2, or tumor-specific upregulation of a particular TRAIL death receptor. In addition, basal TRAIL death receptor membrane expression itself does not seem to correlate with TRAIL sensitivity, indicating the involvement of more downstream mechanisms (38). In this respect, drug-induced effects on other DISC components FADD, caspase 8 and 10, and FLIP have been considered (33). Transcription- and translation-dependent decreases in caspase 8 levels or increases in FLIP levels have both been frequently associated with TRAIL resistance. Numerous drugs have indeed been shown to restore TRAIL sensitivity primarily by increasing caspase 8 levels and/or decreasing FLIP levels (39-41).

In the clinic, rhTRAIL has been combined with rituximab, a CD20-targeting antibody and chemotherapy. The combination of rhTRAIL with rituximab was well tolerated and the maximum tolerated dose was not reached. Twelve patients with relapsed low-grade non-Hodgkin's lymphoma (NHL) were included and three complete tumor responses were observed as well as three partial responses (42). In another clinical trial, rhTRAIL was combined with carboplatin, paclitaxel and bevacizumab, an anti-VEGF antibody. The preliminary data reports of this ongoing study in non-small cell lung cancer (NSCLC) patients showed one complete response and nine partial responses out of twenty four patients (43). The clinical data obtained so far in phase 1 trials demonstrate that rhTRAIL can be administered safely and shows potential as an anti-tumor agent, especially when combined with other therapies.

Table 1. Effects of anti-cancer treatments on TRAIL-R1 and/or TRAIL-R2 expression.

Treatment	TRAIL-R1		TRAIL-R2		Tumor type	Reference
	mRNA	protein	mRNA	protein		
Proteasome inhibitors	n.d.	↑	n.d.	↑	Cervical	(44)
	↑	↑	↑	↑	Prostate	(45)
	n.d.	↑	n.d.	↑↑	Lung	(46)
	n.d.	↑	n.d.	↑↑	Lung	(47)
	n.d.	↑	n.d.	↑	Colon, pancreatic, hepatoma	(48)
HDAC inhibitors	n.d.	=	n.d.	↑	Breast, leukemia	(49,50)
	↑	↑	↑	↑	Breast, myeloma	(51,52)
	=	=	↑	↑	Renal, bladder	(53,54)
	↑	↑	↑	↑	Leukemia	(55)
	n.d.	↓	n.d.	↑	Leukemia	(56)
NSAIDs	=	n.d.	↑	↑	Colon	(57)
	↑	n.d.	↑↑	↑	Prostate	(57)
	n.d.	↑	n.d.	↑	Colon	(58)
	↑	↑	↑	↑↑	Lung	(59)
Irradiation	=	=	↑	↑	Prostate	(60)
	n.d.	=	n.d.	↑	Colon, lung, head and neck	(61)
	n.d.	↑	n.d.	=	Leukemia	(62)
	n.d.	↑	n.d.	↑	Cervix	(63)
	=	=	=	↑	Leukemia	(64)
Topoisomerase inhibitors	↑	=	↑	↑	Renal	(65)
	=	=	↑	↑	Colon	(66)
	n.d.	=	n.d.	↑	Colon, leukemia	(67,68)
	↑	↑	↑	↑	Prostate	(69)
	↑	↑	↑↑	↑↑	Breast	(70)
	↑	n.d.	↑	n.d.	Colon	(71)
	n.d.	=	n.d.	↑	Myeloma	(72)
Cisplatin	n.d.	↑	n.d.	↑	Esophageal	(73)
	n.p.	n.p.	n.d.	↑	Ovarian	(74)
	n.d.	=	n.d.	↑	Lung	(75)
5-FU	n.d.	↑	n.d.	↑↑	Hepatocellular	(76)

Legends: not determined (n.d.), not present (n.p.), upregulation (↑), downregulation (↓), no effect (=), Histone Deacetylase (HDAC), non-steroidal anti-inflammatory drugs (NSAIDs), 5-FU (5-fluorouracil). Bold references represent studies that examined DISC formation.

E. TRAIL combination therapy enhances DISC formation

In order to dissect the underlying mechanisms that contribute to TRAIL sensitization, the efficiency of DISC formation needs to be addressed. Indeed, several studies have shown that the effect of pre-treatment with various therapies not only caused TRAIL-R1/-R2 upregulation but also enhanced DISC formation as described by some examples below (bold references in Table 1).

Irradiation of Jurkat cells overexpressing Bcl-2 slightly induced TRAIL-R2 membrane expression but greatly modulated the TRAIL-DISC as shown by immunoprecipitation (IP) of this complex using flag-tagged TRAIL (64). FADD recruitment to the DISC was four- to five-fold increased. As a consequence, the TRAIL-DISC contained more activated caspase 8 and 10 following irradiation while the level of the long FLIP isoform (FLIP_L) was unchanged, resulting in an higher caspase 8 and 10/FLIP_L ratio (64).

The effect of the proteasome inhibitor bortezomib on TRAIL-induced apoptosis has been examined in hepatocellular, colonic, and pancreatic cancer cell lines (48,77). Both TRAIL-R1 and TRAIL-R2 surface expression were enhanced following bortezomib treatment in all tumor types. TRAIL death receptor upregulation was, however, only partially responsible for TRAIL sensitization since pre-incubation with TRAIL followed by single treatment with bortezomib still sensitized cells. In addition to this, Kyritsis *et al.* showed that TRAIL sensitivity was generally unrelated to TRAIL-R2 expression levels in a panel of eight glioma cell lines but instead was due to FLIP downregulation and caspase dependent Akt inhibition (78). Bortezomib was also used in primary human glioma cells. A collection of TRAIL-resistant primary tumor cells were sensitized to TRAIL by co-treatment with bortezomib (77). Subsequent analysis of the TRAIL-DISC employing biotinylated IZ-TRAIL revealed that pre-treatment with bortezomib increased the caspase 8/FLIP ratio at the DISC (48). On the contrary, proteasome inhibition was found to inhibit TRAIL-induced apoptosis in cervical cancer cells by blocking FLIP and XIAP degradation, which decreased the caspase 8/FLIP ratio at the DISC (79). It was demonstrated that timing of treatment with proteasome inhibitors prior to TRAIL treatment was important; first the proteasome inhibitor plays an anti-apoptotic role by stabilizing FLIP and XIAP but at a later stage it has a pro-apoptotic function by blocking degradation of caspases (79). The opposite effects of proteasome

inhibition depending on the timing of administration reveal the importance of the caspase 8/FLIP ratio at the DISC.

TRAIL-resistant hepatocellular carcinoma cells co-treated with 5-fluorouracil (5-FU) and TRAIL demonstrated synergistic effects on apoptosis induction (76). TRAIL-R2 membrane expression was induced in all cell lines tested, TRAIL-R1 surface expression only in one cell line. The authors showed that TRAIL-R1/-R2 upregulation by 5-FU was not required for TRAIL sensitization since pre-incubation with TRAIL followed by treatment with 5-FU alone still sensitized cells. DISC-IP with biotinylated TRAIL showed that although TRAIL-R1 and TRAIL-R2 levels at the DISC remained similar following 5-FU combination treatment, TRAIL recruited more FADD and caspase 8, and similar levels of FLIP to the DISC in the cells co-treated with 5-FU (76). Colon carcinoma cells were also sensitized to TRAIL by 5-FU and by the chemotherapeutic drugs cisplatin and doxorubicin (74). Again, DISC-IP with flag-tagged TRAIL showed that chemotherapy enhanced FADD and pro-caspase 8 recruitment to the DISC, while recruitment of FLIP was decreased (74). The results of both studies imply that treatment with various drugs can result in a higher caspase 8/FLIP ratio at the TRAIL-DISC.

Histone deacetylase (HDAC) inhibitors have been shown to affect TRAIL-DISC formation. In both renal cell carcinoma and bladder tumor cells, TRAIL sensitization was accompanied by enhanced TRAIL-R2 but not TRAIL-R1 surface expression. Treatment of cells with TRAIL and HDAC inhibitors increased TRAIL-R2 levels and enhanced FADD recruitment to the DISC as demonstrated by DISC-IP with flag-tagged TRAIL (53,54). Both TRAIL-R1 and TRAIL-R2 protein and mRNA levels were upregulated by the HDAC inhibitor LAQ284 in acute myelocytic leukemia (AML) cells (55). Membrane expression of TRAIL-R2 and to a lesser extent of TRAIL-R1 was induced, suggesting that HDAC inhibitors are not TRAIL-R1 specific. DISC-IP with a TRAIL-R1 antibody showed that pre-treatment with LAQ284 increased caspase 8 and FADD levels at the DISC, while FLIP levels were unchanged (55). Similarly, other HDAC inhibitors had a synergistic effect when combined with TRAIL in chronic lymphocytic leukemia (CLL) cells (56). TRAIL-R2 surface expression levels were only slightly affected. DISC-IP with biotinylated TRAIL showed that TRAIL-R2, FADD and caspase 8 recruitment to the DISC was increased following pretreatment with HDAC inhibitors, resulting in a higher caspase 8/FLIP ratio at the DISC (56).

These studies clearly demonstrate that irradiation- or drug-induced TRAIL sensitization facilitate the formation of DISCs and is a main factor in combined treatment sensitization. It is noteworthy that in all these studies biotinylated or flag-tagged TRAIL was used to immunoprecipitate the DISC and these tagged ligands are known to be both more potent than un-tagged TRAIL and toxic to normal cells (25). It remains to be elucidated whether these DISC-IP findings are representative for signaling induced by non-tagged TRAIL. In order to fully exploit the apoptotic effect of TRAIL or combination treatment with TRAIL, we should gain more insight into the mechanisms underlying TRAIL sensitivity and drug-induced TRAIL sensitization in relation to DISC formation. The studies mentioned above demonstrate that the caspase 8/FLIP ratio at the DISC is unequivocally important for effective TRAIL signaling. Apart from differences in caspase 8/FLIP ratios at the DISC, other factors have been described to influence DISC formation and these will be addressed in the next section.

F. Loss of TRAIL-R1 and TRAIL-R2 expression

The genes coding for the four membrane TRAIL receptors are located on the 8p21-22 chromosome (80-82). This chromosome is a hot-spot for deletions and is frequently subject to allelic loss in various cancer types, including non-Hodgkin's lymphoma and lung, colon, breast, prostate, hepatocellular, ovarian, and head and neck cancer (83-92). Epigenetic silencing of TRAIL-R1 and/or TRAIL-R2 has been reported in melanoma and ovarian cancer (93,94). Because of monoallelic deletion, frequent downregulation of these TRAIL death receptors has been found in primary B-cell non-Hodgkin's lymphoma (B-NHL) (95). TRAIL resistance was correlated with 8p chromosome deletion and reduced TRAIL death receptor expression in most lymphoma cell lines tested. Enhancing TRAIL-R1 or TRAIL-R2 expression by less than two-fold re-sensitized these cells to TRAIL, which suggests a gene-dosage effect of these deletions. In the FaDu nasopharyngeal cancer cell line, homozygous loss of TRAIL-R1 has led to TRAIL resistance, while re-expression of TRAIL-R1 could again sensitize this cell line to TRAIL (96). Similar results were seen in the ovarian cancer line A2780, where reestablishing TRAIL-R1 expression with the demethylating agent 5-aza-2'-deoxycytidine or transient transfection with a full-length TRAIL-R1 construct restored sensitivity to TRAIL (94). It is noteworthy that TRAIL resistance in A2780 cells could also be overcome by specific

targeting of TRAIL-R2 with a TRAIL-R2-specific form of TRAIL or combination with cisplatin, both *in vitro* and *in vivo* (97). Additional data indicate that membrane TRAIL-R1 levels correlate with TRAIL sensitivity in different cancer cell types (98-100). Conversely, studies showed that TRAIL death receptor expression generally tends to increase during carcinogenesis, and tumors often express at least one TRAIL death receptor (72,101,102). Thus, although complete loss of both TRAIL-R1 and TRAIL-R2 might not be frequent, loss of one TRAIL death receptor only is sufficient to induce TRAIL resistance. In cells that have lost one of the TRAIL death receptor, combination therapies that either increase TRAIL-signaling via the remaining receptor or reestablish expression of the silenced receptor can restore TRAIL sensitivity.

Whereas mice lacking TRAIL are viable, both caspase 8 and FADD are crucial to embryonic development and normal lymphocyte proliferation (103). These findings make complete loss of expression of these proteins less likely than loss of TRAIL death receptors.

MiRNAs are a family of small (18-25nt) non-coding RNAs that regulate protein expression at a post-transcriptional level via binding to specific mRNAs. Recently, several screens have been performed to identify miRs that are involved in TRAIL signaling and sensitivity in cancer (104,105). One of these screens identified miRs that regulate expression of DISC components (105). miR-145 and -216 were predicted to target TRAIL-R1 and TRAIL-R2, while FADD was a predicted target of MiR-182 and -96 (105). Although these studies are preliminary, miRNAs affecting DISC components could constitute interesting markers for TRAIL death receptor-mediated sensitivity and/or drug targets in the future.

G. DISC component polymorphisms and functionality

Besides loss of TRAIL-R1 or TRAIL-R2 expression in tumor cells, alterations in the TRAIL death receptor gene sequences might play a role in TRAIL sensitivity (see Table 2).

In hematological tumors, a variant of TRAIL-R1 with a single-nucleotide polymorphism within the extracellular cysteine-rich domain was associated with several types of cancer, including CLL and mantle cell lymphoma (MCL) (106). This is particularly interesting since TRAIL-induced apoptosis was shown to depend on

TRAIL-R1 in CLL cells (107). Several other polymorphisms leading to codon changes, often found in the DD or the cysteine-rich domain of TRAIL-R1 or TRAIL-R2, have also been associated with enhanced risk for MCL and B-cell lymphoid neoplasms (108). Since these cancers could, in part, develop due to inefficient TRAIL receptor-mediated signaling, they might also respond poorly to TRAIL death receptor-targeted therapies.

In solid tumors, two missense polymorphisms potentially affecting TRAIL binding were found to co-segregate in the extracellular domain of TRAIL-R1 in primary NSCLC, gastric adenocarcinoma and in head and neck squamous cell cancer (HNSCC). These polymorphisms were also present in matched normal tissues and therefore do not constitute mutations per se. However, the frequency of being homozygous for these two polymorphisms was increased in the cancer samples compared to matched controls, suggesting a predisposition to cancer (109). Polymorphic alterations in TRAIL-R1, mostly in the extracellular cysteine-rich domain of the receptor, were increased in osteosarcoma, but also in prostate cancer metastases (110,111). TRAIL-R2 mutations were found in HNSCC, NSCLC, breast cancers, hepatocellular carcinomas and gastric cancers, often in the exon coding for the DD (exon 9) (112-116)(Table 2).

In metastatic breast cancers, Shin *et al.* found at least four TRAIL-R2 mutations that interfere with apoptosis transduction in metastatic lesions, while three other mutations were both present in the primary tumors and in the metastatic lesions (116). Lack or truncation of the DD of TRAIL-R2 was reported in a sporadic colon cancer patient with liver metastasis and in the RT4 bladder cancer cell line, respectively (118,119).

Several of the TRAIL-R1 and TRAIL-R2 mutations have been found in conserved hydrophobic residues and charged amino acids of the DD in cancer cells (113) where they hampered efficient DISC recruitment of FADD and caspase 8 (120). Bin *et al.* recently tested four additional point mutations frequently found in the DD of TRAIL-R2 in cancer cells (121). Although his-tagged TRAIL could still bind to mutated TRAIL death receptors, efficient DISC formation was hindered, and TRAIL sensitivity was decreased. His-tagged TRAIL treatment did not induce formation of inactive wild type TRAIL-R1/mutant TRAIL-R2 heterotrimers. It is therefore unclear why excess concentrations of his-tagged TRAIL could not restore TRAIL sensitivity by activating the functional TRAIL-R1. Although this could be partially due to competition between the overexpressed mutant TRAIL-R2 and endogenous TRAIL-R1, it is interesting that

TRAIL signaling was less hampered by TRAIL-R2 loss than by TRAIL-R2 mutations. Only TRAIL-R1-selective agonists could overcome the effects of mutations in TRAIL-R2 and induce apoptosis. Given the presence of TRAIL death receptor mutations in circa 10% of human tumors, prior screening could therefore be critical for the choice of ligands. Furthermore, although the negative effects of TRAIL-R1 or TRAIL-R2 mutations on DISC formation have been described, the consequences on TRAIL receptor-mediated pro-survival signaling still need to be addressed.

Table 2. Mutations found in TRAIL-R1 and TRAIL-R2 in various cancers.

Tumor type	Patients (n) screened for TRAIL-R1 mutations	Mutations observed in TRAIL-R1 (location)	Patients (n) screened for TRAIL-R2 mutations	Mutations observed in TRAIL-R2 (location)	Reference
B-NHL	117	2 (DD)	117	6 (4 different mutations in the DD, 1 truncating mutation before the DD)	(117)
HNSC	n.d.	n.d.	60	2 (DD, incl. 1 germline truncating mutation)	(114)
NSCLC	n.d.	n.d.	104	11 (10 in the DD, 1 in intro 8)	(113)
Breast cancer	57	3 (DD)	57	4 (2 DD + 2 in DD flanking regions)	(116)
Gastric cancer	n.d.	n.d.	43	3	(115)
HCC	n.d.	n.d.	100/DD*	1 (DD)	(112)
			incl. 40/entire gene	None additional	(112)

Legends: not determined (n.d.), head and neck squamous cell cancer (HNSC), non-small cell lung cancer (NSCLC), B-cell non-Hodgkin's lymphoma (B-NHL), hepatocellular cancer (HCC). * Only the DD-coding region was analyzed.

It should also be noted that some rare polymorphisms and mutations have been reported in the genes coding for caspase 8 and FADD (122,123). In comparison, TRAIL-R1 and TRAIL-R2 gene alterations have been found in a significant number of tumors. Without screening options to determine functionality of either TRAIL-R1 or TRAIL-R2, a combination of TRAIL-R1 and TRAIL-R2-specific ligands might be a strategy to increase the likelihood of activating the TRAIL death receptor(s) and prevent eventual decoupling effects due to the presence of a non-functional receptor.

H. Death receptor-selective TRAIL variants

As it has been shown that in CLL cells TRAIL specifically signals through TRAIL-R1 (107), the generation of TRAIL variants with a higher affinity for TRAIL-R1 and/or TRAIL-R2 has raised interest in order to enhance DISC formation. Several TRAIL-R1- and TRAIL-R2-selective TRAIL variants were designed using computational design (124-126). Two TRAIL-R1-selective forms of TRAIL were generated with a single amino acid mutation at position 218 from asparagine to either histidine or tyrosine in the receptor binding domain. Binding assays revealed that both TRAIL-R1-selective forms had a decreased affinity for TRAIL-R2 and TRAIL decoy receptors as compared to normal TRAIL, while their affinity for TRAIL-R1 was unchanged. The TRAIL-R1-selective TRAIL variants effectively induced apoptosis in cell lines that specifically signal via TRAIL-R1 whereas almost no apoptotic effect was induced in cells expressing exclusively TRAIL-R2. However, the TRAIL-R1-selective variants were less effective in TRAIL-R1-responsive cells when compared to TRAIL (124). Therefore, new TRAIL variants with increased affinity to both TRAIL-R1 and TRAIL-R2 were designed, by replacing glycine-131 by lysine or arginine (126). The efficacy of these TRAIL variants was compared to the efficacy of TRAIL in a panel of cell lines that signal TRAIL induced apoptosis via TRAIL-R1, TRAIL-R2 or both receptors, and demonstrated an increased apoptotic effect in all cell lines regardless of their TRAIL death receptor signaling status (126). Using the same computational design, van der Sloot *et al.* generated a TRAIL-R2-selective TRAIL variant with mutations D269H and E195R, resulting in largely reduced affinity for TRAIL-R1, less affinity to TRAIL decoy receptors, but higher affinity for TRAIL-R2. This TRAIL-R2-selective variant induced more apoptosis than TRAIL in colon carcinoma, ovarian carcinoma and chronic myeloid

leukemia (CML) cells, while TRAIL-R1-selective cells were only sensitive to TRAIL (125). Upon combination with cisplatin, the TRAIL-R2-selective TRAIL variant showed enhanced anti-tumor efficacy as compared to rhTRAIL both *in vitro* and in a bioluminescent ovarian cancer xenograft model (97).

Kelley *et al.* generated TRAIL death receptor-selective TRAIL mutants using a phage display approach (127). The TRAIL-R1-binding TRAIL mutants carried an Y189A mutation and had increased affinity for TRAIL-R1, while binding to TRAIL-R2 was undetectable. Mutations Q193K and Q193R in TRAIL proved to be important for its affinity for TRAIL-R2. In apoptosis assays, the TRAIL-R1-selective mutants were less potent as compared to flag-tagged TRAIL in lung carcinoma cells, whereas the TRAIL-R2-selective mutants retained their apoptosis-inducing ability. However, the potency of the TRAIL-R1-selective mutants was not verified in cell lines exclusively expressing TRAIL-R1. MacFarlane *et al.* (128) demonstrated that the TRAIL-R1-selective variants described by Kelly *et al.* (127) did not induce apoptosis in Ramos cells, which were previously shown to signal via TRAIL-R1 (107). This strengthens the possibility that the mutations in the TRAIL-R1-selective TRAIL variant caused a loss in apoptotic potency. Therefore, the authors synthesized several new TRAIL-R1-selective TRAIL mutants. One TRAIL variant with five mutations (Q193S, N199V, K201R, Y213W, and S215D) induced less apoptosis in TRAIL-R2-positive Jurkat cells as compared to TRAIL, while its TRAIL-R1-specific activity in Ramos cells was preserved. In addition, primary CLL and MCL cells were sensitive to treatment with the TRAIL-R1-selective variant. DISC-IPs in cells treated with this variant demonstrated enhanced recruitment of FADD and caspase 8 to the DISC, as compared to DISC-IPs after TRAIL exposure. This suggests that specific activation of the dominantly signaling TRAIL death receptor by a selective TRAIL mutant results in enhanced DISC formation and apoptosis induction as compared to the use of TRAIL that binds both TRAIL death receptors.

It is currently not possible to determine which TRAIL death receptor predominantly transduces the apoptosis signal in a given primary tumor. However, the development of TRAIL-R1- and TRAIL-R2-selective TRAIL variants opens ways to circumvent the TRAIL decoy receptors and warrant activation of the dominant TRAIL death receptor when used in combination.

I. TRAIL death receptor-specific agonistic antibodies

In addition to the receptor-selective TRAIL variants, TRAIL-R1- and TRAIL-R2-specific agonistic antibodies have been developed. One important difference between the agonistic antibodies and rhTRAIL is the extended serum half-life of the antibodies in men, which is around 2-2.5 weeks whereas it is 25 min for rhTRAIL (129,130).

HGS-ETR1 (mapatumumab) is the only anti-TRAIL-R1 antibody currently in clinical development, while five anti-TRAIL-R2 antibodies are being tested, namely HGS-ETR2 (lexatumumab), Apomab (PRO95780), TRA-8 (CS-1008), AMG655 and LBY135. These agonistic antibodies effectively induced apoptosis in a broad panel of cancer cell lines, without toxicity to normal hepatocytes (131-139). Cross-linking of the anti-TRAIL-R2 antibodies was either required (TRA-8), or beneficial (HGS-ETR2 and Apomab) for their apoptotic effect *in vitro* (132,137,138). Apomab, TRA-8 and LBY135 were shown to inhibit tumor cell growth in xenograft mice models (137-140). TRA-8 also induced cell death in *ex vivo* tissue slice models with primary human ovarian and cervical cancer tissue (141,142).

Combination of chemotherapeutic drugs with the TRAIL death receptor-specific agonistic antibodies or TRAIL variants may show whether a drug specifically sensitizes cells via TRAIL-R1 or TRAIL-R2. Bortezomib was combined *in vitro* with both HGS-ETR1 and HGS-ETR2 which resulted in additive or synergistic effects on (apoptotic) cell death in leukemic and lymphoma cells (133,136). Bortezomib treatment also sensitized a panel of NSCLC cells to both HGS-ETR1 and HGS-ETR2, while cells were resistant to treatment with the antibodies alone. Remarkably, bortezomib only induced expression of TRAIL-R2 while TRAIL-R1 levels remained the same. Sensitization to HGS-ETR1 can thus not be attributed to TRAIL-R1 upregulation (143). The effect of radiation on the sensitivity for different antibodies was also investigated. In colon carcinoma cell lines, increased sensitivity to HGS-ETR1 and HGS-ETR2 was seen following irradiation (134). In cervical cell lines, radiation was found to sensitize cells to TRAIL via TRAIL-R1, while radiation sensitized cells to both TRAIL-R1 and TRAIL-R2 antibodies (63). In addition, cervical and breast cancer cells were successfully sensitized to the anti-TRAIL-R2 antibody TRA-8 *in vitro* as well as *in vivo* (144,145). Doxorubicin was shown to sensitize primary lymphoma cells to both HGS-ETR1 and HGS-ETR2 *in vitro*, and breast cancer cells to TRA-8 *in vitro* as well as *in vivo* (133,144). These examples of studies combining

different drugs or radiation with agonistic antibodies against TRAIL-R1 or TRAIL-R2 further suggest that the observed sensitization is not TRAIL death receptor specific.

TRAIL death receptor-specific antibodies are currently under investigation in several phase 1 and 2 clinical trials, as single agents or in combination with various chemotherapeutics (129,130,146-160). Tumor responses were observed in 3 out of 40 extensively pretreated NHL patients (one complete and two partial responses) on anti-TRAIL-R1 antibody (151). The antibodies are well-tolerated, also when combined with chemotherapy, and may be particularly interesting when used in combination with other anti-tumor agents. An overview of the anti-TRAIL-R1 and anti-TRAIL-R2 agonistic antibodies under clinical investigation, and of particular interest in combination with other anti-tumor agents, was recently presented by Oldenhuis *et al.* (14).

J. Regulation of TRAIL death receptor-mediated apoptotic signaling at the cell membrane

The caspase cascade can be strongly influenced by membrane-proximal events, independent of TRAIL death receptor or caspase 8 expression levels. Several factors contributing to efficient DISC recruitment and apoptosis have been described.

1. TRAIL-R1 and TRAIL-R2 aggregation promotes DISC formation

TRAIL death receptors can trimerize independently of the presence of TRAIL (161), but stimulation by oligomers of at least 3 ligands is required for DISC activation (21). Unlike TRAIL, anti-TRAIL-R1 or anti-TRAIL-R2 agonistic antibodies bind only two receptor subunits and as a consequence these ligands possess different abilities to trigger TRAIL death receptor oligomerization. A study by Chuntharapai *et al.* established that mouse anti-human TRAIL-R1 antibodies require cross-linking to be effective in a variety of cancer cell lines *in vitro* (162). *In vivo*, these antibodies demonstrated efficacy in different human xenograft mouse models, even in absence of the exogenous cross-linking agents that are essential for their activity *in vitro*. These results support the existence of an endogenous oligomerization mechanism, which based on *in vitro* and *in vivo* experiments are independent of both the antibody-dependent cell-mediated cytotoxicity and the complement-dependent cytotoxicity (139,162).

Remarkable discrepancies have been found between TRAIL-R1 and TRAIL-R2 requirement for ligand-induced aggregation. TRAIL-R2 only responded to membrane-bound or cross-linked soluble flag-tagged TRAIL, whereas soluble flag-tagged TRAIL (sTRAIL) was sufficient to induce TRAIL-R1-mediated apoptosis (163,164). Not all TRAIL preparations require aggregation to activate TRAIL-R2 since rhTRAIL can stimulate this receptor without cross-linking (125,165). However, the results obtained with sTRAIL suggest that ligand aggregation is more important for TRAIL-R2 than for TRAIL-R1. It is noteworthy that the potency of sTRAIL, anti-TRAIL-R1 and anti-TRAIL-R2 antibodies was enhanced upon ligand pre-aggregation, which indicates that aggregation can be beneficial to signaling via both TRAIL-R1 and TRAIL-R2 (163). The superior importance of ligand cross-linking for TRAIL-R2 compared to TRAIL-R1 was further demonstrated with anti-TRAIL death receptor agonistic antibodies. Apoptotic signaling could only be initiated by cross-linked anti-TRAIL-R2 antibodies in CLL cells, whereas anti-TRAIL-R1 antibodies induced apoptosis in absence of cross-linking. Accordingly, TRAIL-induced apoptosis signaling was predominantly mediated by TRAIL-R1 in these cells (166). It is remarkable that HGS-TR2J, an anti-TRAIL-R2 agonistic antibody, induced apoptosis in absence of cross-linking (direct agonist) and was more efficient than its cross-linking-dependent agonist counterpart HGS-ETR2 in a xenograft model. This indicates that binding to specific sites in the extracellular domain of TRAIL-R2 can induce sufficient aggregation (167). Unfortunately, no clinical trials have been reported for HGS-TR2J (<http://clinicaltrials.gov/ct2/home>). Finally, the possibility that excessive aggregation might enhance toxicity in normal cells should be further evaluated in order to design optimal therapeutics. His-tagged and flag-tagged TRAIL, crosslinked or not with an antibody, tend to aggregate as opposed to non-tagged soluble trimeric forms such as LZ- or rh-TRAIL. The presence of aggregates that would induce excessive aggregation of TRAIL-R1 and TRAIL-R2 could explain the toxicity of these tagged forms of TRAIL toward normal cell types as opposed to stable TRAIL trimers (25,165,168).

In addition to the epitopes recognized by the anti-TRAIL-R1 or anti-TRAIL-R2 antibodies, studies have shown that the antibody isotypes should also be taken into account *in vivo*. Because of the strong aggregating properties of these isotypes, IgM and IgG3 antibodies raised against CD95 exhibited liver toxicity (13). Murine IgG2a isotype antibodies have been reported to be more potent than murine IgG1 isotype antibodies

for inducing complement-dependent death receptor oligomerization *in vivo* (169). Although both murine anti-TRAIL-R1 isotypes exhibit similar apoptosis induction *in vitro*, mouse IgG1 anti-human TRAIL-R1 antibodies are unexpectedly more effective in human xenograft mouse models than the IgG2 variants (162). Altogether, these findings highlight the need for further studies on the contribution of the immune response to the potency of the various isotypes targeting the same TRAIL death receptor *in vivo*.

Although aggregation of the TRAIL death receptors can be induced via ligand binding, various drugs have also been shown to stimulate receptor aggregation. The bile acid glycochenodeoxycholic acid (GCDCA) increased TRAIL-R2 expression and aggregation in a punctuated pattern in a hepatocellular cancer cell line but this was not seen for TRAIL-R1. These changes were associated with an increase in TRAIL sensitivity (170). Similarly, LY303511, an inactive analogue of the phosphoinositide-3 kinase (PI3K) inhibitor LY294002, induced ligand-independent oligomerization of TRAIL-R2 in high molecular weight TRAIL-R2 complexes (200-400 kDa) and stimulated TRAIL-induced apoptosis. Imaging by confocal microscopy showed a more patchy distribution of TRAIL-R2 in LY303511-treated cells, which was coupled to higher FADD and caspase 8 recruitment at the DISC (171).

2. Lipid raft localization stimulates TRAIL death receptor aggregation

The presence of TRAIL death receptors in lipid rafts on the cell membrane constitutes another essential mechanism for efficient TRAIL signaling. The term “lipid rafts” typically refers to insoluble detergent (often Triton X-100)-resistant membrane domains rich in sphingomyelin, sphingolipids and cholesterol (172-174). Proteins involved in various cellular signaling pathways, notably Src kinases, have been shown to distinctively associate with these lipid rafts, facilitating signal modulation (172,175). Numerous studies performed over the past 10 years have indicated that translocation of CD95 into lipid rafts is very important for its ligand sensitivity since it promotes receptor clustering and aggregation (176). Recent evidence now indicates that membrane rafts also play a crucial role in the regulation of TRAIL-induced cell death. TRAIL, alone or in combination with several chemotherapeutic agents, stimulates DISC formation by relocating DISC proteins into lipid rafts (Table 3).

Table 3. TRAIL receptors and lipid rafts.

Agents promoting TRAIL-R1/-R2 lipid rafts localization	Agent inhibiting TRAIL-R1/-R2 lipid raft localization	DISC proteins recruited to the lipid raft fraction	Lipid raft marker used	Cell type	Reference
Edelfosine, perifosine	MCD, filipin	TRAIL-R1/-R2, FADD, caspase 8	GM1	Multiple myeloma	(177)
Doxorubicin	M β CD	TRAIL-R2	Ceramide	Lymphoma cells, T splenocytes	(178)
Resveratrol	nystatin	TRAIL-R1/-R2, caspase 8, FADD	Caveolin-2	Colon cancer	(179)
Depsipeptide	nystatin	TRAIL-R1/-R2	Lck	Prostate cancer	(180)
rhTRAIL, cisplatin	M β CD	TRAIL-R1/-R2, FADD, caspase 8	Caveolin-1	NSCLC	(75)
Apo-2L/TRAIL + TRAIL enhancer (crosslinker)	n.d.	TRAIL-R2, caspase 8	Caveolin-1	Glioma	(181)
Quercetin	nystatin	TRAIL-R1/-R2, FADD, caspase 8	Caveolin-1	Colon cancer	(182)
DuP-697, acid arachidonic	M β CD, imipramine	TRAIL-R1/-R2, FADD, caspase 8	Ceramide, caveolin-1, flotilin	Colon cancer	(183)
Roscovitine, TRAIL	M β CD, imipramine	TRAIL-R2, FADD, caspase 8	n.d.	Breast cancer	(184)
Oxaliplatin	nystatin	TRAIL-R1/-R2	Ceramide	Gastric cancer	(185)

Legends: not determined (n.d.), nystatin (cholesterol-sequestering agent), depsipeptide (FR901228, HDAC inhibitor), methyl-cyclodextrin (MCD, cholesterol-depleting agent), methyl- β -cyclodextrin (M β CD, cholesterol-depleting agent), filipin (cholesterol-sequestering agent), edelfosine and perifosine (synthetic alkyl-lysophospholipids), ganglioside (GM1), imipramine (acid sphingomyelinase inhibitor).

Most studies have shown that lipid raft disruption leads to a loss of the sensitizing effects of these chemotherapeutic drugs, or to a general loss of TRAIL sensitivity. Work by Song *et al.* revealed that DISC formation could also take place in

non-raft fractions. However, the importance of the lipid rafts for effective TRAIL death receptor signaling was highlighted by the fact that their disruption with methyl- β -cyclodextrin (M β CD) inhibited both caspase 8 cleavage and TRAIL-induced cell death in TRAIL-sensitive cells. Furthermore, in TRAIL-resistant NSCLC cells, TRAIL failed to recruit caspase 8 and FADD into lipid rafts, suggesting that lipid raft localization correlates with functional DISC formation (75).

Although the molecular mechanisms behind TRAIL death receptor clustering into lipid rafts are not yet entirely clear, an intricate signaling cascade has already been established (see Figure 1). TRAIL stimulates accumulation of arachidonic acid by inducing release of reactive oxygen species (ROS) (186). This event induces translocation of acid sphingomyelinase (ASM) to the plasma membrane and activation of this enzyme, leading to ceramide production. Ceramide-rich caveolae are subsequently formed within the outer leaflet of the plasma membrane (183,186). These membrane domains promote TRAIL-R2 clustering and thereby enhance TRAIL sensitivity (183,186). Transient release of ceramide and clustering of TRAIL-R2 were seen in different types of ASM-positive cells, including cancer cells but not in ASM-negative splenocytes (186). The importance of this initial redox mechanism is supported by the fact that its inhibition by antioxidants such as N-acetylcysteine or Tiron blocks both ASM activation and TRAIL-induced apoptosis (186). Cyclooxygenase-2 (COX-2), which uses acid arachidonic as a substrate, prevents arachidonic acid accumulation, activation of ASM and TRAIL-induced apoptosis. Downregulation of COX-2 or addition of exogenous C16-ceramide can recapitulate these sensitizing effects, while inhibition of ASM with imipramine or lipid raft destruction with M β CD abolishes TRAIL-R2 clustering in ceramide-rich domains (183). These findings underscore the importance of ASM-dependent lipid raft formation for TRAIL sensitivity.

Martin *et al.* raised the possibility that type I and type II cells might have different lipid raft localization patterns. In type I cells, TRAIL-R2 and the DISC components were pre-existent within lipid rafts at the plasma membrane. In type II cells, pre-incubation with DuP697 was required for these proteins to colocalize. Interestingly, it was shown in glioma cell lines that TRAIL-induced translocation of TRAIL-R1 or TRAIL-R2 concomitantly stimulates TNF-R1 and CD95 translocation into lipid rafts, suggesting a possible cross-modulation of these receptors (181).

Model for TRAIL-R1/-R2 aggregation into lipid rafts

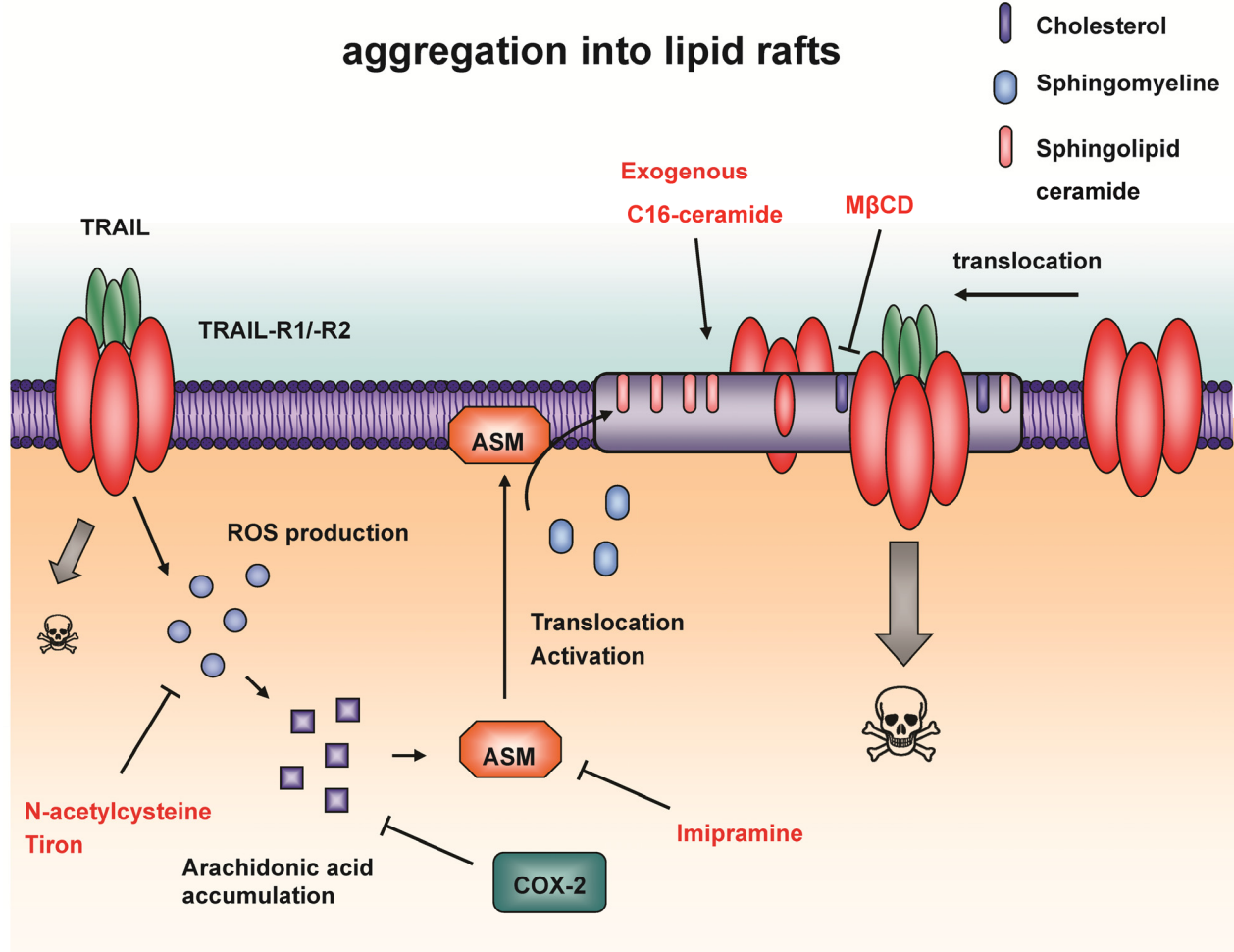


Figure 1: Model for TRAIL-R1/-R2 aggregation into lipid rafts. Upon binding of TRAIL to its TRAIL death receptors, little pro-apoptotic DISCs are formed. However, a rapid release of ROS causes arachidonic acid accumulation, which itself induces translocation of ASM to the plasma membrane and stimulates ASM activity. This leads to the production of sphingolipid ceramide in the outer leaflet of the cell membrane, initiating formation of ceramide-rich caveolae. These membrane structures, also called lipid rafts, promote aggregation of the TRAIL receptors. TRAIL death receptor aggregation within lipid rafts induces more efficient DISC formation, caspase cleavage and higher levels of apoptosis. Antioxidants such as N-acetylcysteine and Tiron can inhibit ROS production and lipid rafts formation. COX-2, by inhibiting the accumulation of arachidonic acid, inhibits acid sphingomyelinase activity as well as consequent TRAIL receptors aggregation in lipid rafts and apoptosis. TRAIL receptor aggregation and apoptosis can also be inhibited by imipramine, an ASM inhibitor. Lipid rafts can be disrupted by the cholesterol depleting agent β -MCD. Conversely, inhibition of COX-2 or addition of exogenous C16-ceramide stimulates TRAIL-induced apoptosis.

Drug-induced lipid raft localization of TRAIL death receptors may not always contribute to the observed TRAIL sensitization. In breast cancer cells, the cyclin-dependent kinase 2 inhibitor roscovitine increased the amounts of procaspase 8, FADD and TRAIL-R2 in the lipid raft fraction, DISC formation was enhanced, and cells were sensitized to TRAIL. However, lipid raft localization did not seem to contribute to the sensitizing effect of roscovitine as neither the cholesterol disrupting agent M β CD nor the ASM inhibitor imipramine were able to protect cells from the TRAIL-sensitizing effect of roscovitine (184). Importantly, predominant localization of TRAIL-R1 or TRAIL-R2 outside of lipid rafts can induce pro-survival signals, thereby adding even more complexity. In TRAIL-resistant NSCLC cell lines, TRAIL treatment only resulted in activation of the nuclear factor- κ B and the extracellular signal-regulated kinase 1/2 (ERK1/2) survival pathways. In these TRAIL-resistant cells, TRAIL-R1 and TRAIL-R2 were primarily located outside of the raft fractions. Recruitment of Receptor-interacting protein (RIP) and FLIP at the DISC played a crucial role in both DISC assembly in the non-raft fraction and downstream stimulation of the survival pathways (75).

Interestingly, although extensive data have been collected on the relation between lipid raft localization of TRAIL death receptors and apoptotic signal transduction, little is known about the recruitment of TRAIL decoy receptors to the lipid rafts, and its consequences for apoptosis. In various cell types, including cancer cells, TRAIL-R3 and TRAIL-R4 were mostly found in lipid rafts, where they competitively inhibited TRAIL binding to the TRAIL death receptors and consequently decreased TRAIL sensitivity (32). The influence of the lipid rafts on this decoupling effect of TRAIL decoy receptors has yet to be evaluated in a non-overexpression model. Future experiments are needed to determine whether TRAIL sensitizers directing TRAIL-R1 and TRAIL-R2 to membrane rafts also have stimulating effects on TRAIL-R3 and TRAIL-R4, which could at the same time dampen the pro-apoptotic effects of these drugs. If so, TRAIL-R1- or TRAIL-R2-specific agonists might potentially circumvent this problem.

TRAIL death receptor localization seems to be regulated in a receptor-specific and cell-specific manner. Many studies have focused on TRAIL-R2, and have shown that signaling via this receptor depends on lipid raft localization. Type I cells, characterized by efficient DISC formation, tend to show more frequent TRAIL death receptor co-localization within lipid rafts than type II cells. Many cytotoxic agents that enhance TRAIL sensitivity stimulate the recruitment of DISC proteins to membrane rafts. It is

noteworthy that within this new research field, the markers used to define membrane rafts often differ between studies. Results obtained following lipid raft isolation may depend largely on the type and concentrations of detergent used to extract the lipidic detergent-resistant fractions (187). Considering the exciting discoveries made during the last few years, further studies on the relationship between DISC formation, lipid rafts and the kinases found in these rafts are warranted. Upcoming studies should now greatly benefit from recent efforts to establish a consensus definition for these membrane rafts, for instance assessed by the Keystone symposium on lipid rafts and cell function (188).

3. Receptor internalization hampers TRAIL death receptor-mediated apoptosis

Receptor internalization plays a crucial role in TNF family member receptor signaling since this event is required for both TNF-R1- and CD95-mediated apoptotic signaling (189,190). Studies on the importance of TRAIL death receptor internalization have also been performed, with contrasting conclusions compared to TNF-R1 and CD95. TRAIL death receptor internalization, which was shown to be clathrin-dependent, does not seem to be necessary for TRAIL-induced apoptosis (191). In fact, caspase 8 cleavage was slightly enhanced when TRAIL death receptor internalization was blocked (191,192). TRAIL death receptor stimulation was found to disrupt the endocytosis machinery, possibly amplifying caspase cleavage at the DISC (191). In addition, it has been suggested that clathrin-independent mechanisms may also participate in TRAIL death receptor internalization (192). These substantial discrepancies between CD95 (or TNF-R1) and TRAIL death receptor internalization are intriguing, and constitute one of the most striking differences between these pathways. In addition to TRAIL-induced internalization, TRAIL death receptors can also undergo constitutive endocytosis as part of a desensitizing mechanism, in leukemia as well as colon and breast cancer cells (98,100,193). This downregulation of the TRAIL death receptor was clathrin-dependent. Both TRAIL death receptor expression and TRAIL sensitivity could be restored by a general endocytosis inhibitor, phenylarsine oxide (PAO), by an inhibitor of clathrin-mediated apoptosis, chlorpromazine, and by specific gene silencing of either adaptator protein 2 or clathrin (100). TRAIL-R1 was found to be internalized at a higher frequency than TRAIL-R2 despite similar mRNA and protein levels, which suggests an important role for TRAIL-R1 endocytosis in TRAIL resistance (98,100,193).

K. Post-translational modifications of the DISC components

While the contribution of TRAIL death receptor upregulation to TRAIL sensitivity enhancement in response to chemotherapeutic drugs is still debated, it is clear that the caspase 8/FLIP ratio is one of the key factors influencing TRAIL sensitivity. Now, numerous post-translational modifications of the DISC proteins are also emerging as important mechanisms regulating TRAIL sensitivity in cancer cells (see Figure 2 and 3).

1. *TRAIL death receptor O-glycosylation facilitates receptor aggregation*

In connection with TRAIL death receptor aggregation, post-translational modifications of TRAIL-R1 and TRAIL-R2 such as receptor glycosylation have been shown to affect TRAIL signaling, thereby affecting TRAIL sensitivity (see Figure 2). O-linked glycosylation involves the binding of glycosyl groups to serine and threonine side chains that can improve ligand binding and membrane protein activation (194). Wagner *et al.* nicely demonstrated that specific O-glycosyltransferases were overexpressed in cancer tissue as compared to normal tissue and that their expression correlated with TRAIL sensitivity (38). Expression of the O-glycosylation initiating enzyme GALNT14 was correlated with TRAIL sensitivity in pancreatic carcinoma, NSCLC cells and melanoma cells. In colon carcinoma, expression of GALNT3 and of the O-glycan-processing enzymes FUT 3 and FUT 6 correlated with TRAIL sensitivity. Inhibition of O-glycosylation enzymes by benzyl- α -GalNAc decreased TRAIL-induced apoptosis. SiRNA against GALNT14, GALNT3, FUT3, and FUT6 also reduced TRAIL sensitivity while overexpression of GALNT14 increased DISC formation and sensitized cells to TRAIL. Mass spectrometry of the extracellular domain of TRAIL-R2 indicated that TRAIL-R2 could be a direct target of GALNT14. It was suggested that O-glycosylation modulates TRAIL sensitivity by inducing TRAIL death receptor aggregation, thereby promoting DISC formation and caspase 8 activation (38).

2. *TRAIL-R1 S-nitrosylation stimulates caspase 8 cleavage*

S-nitrosylation refers to the covalent binding of a nitrogen monoxide group to a reactive thiol cysteine. This emerging post-translational protein modification regulates a wide range of signal transduction mechanisms, including apoptosis (195). TRAIL-R1 but not TRAIL-R2 was found to be S-nitrosylated following treatment with the nitric oxide-

The “good” DISC

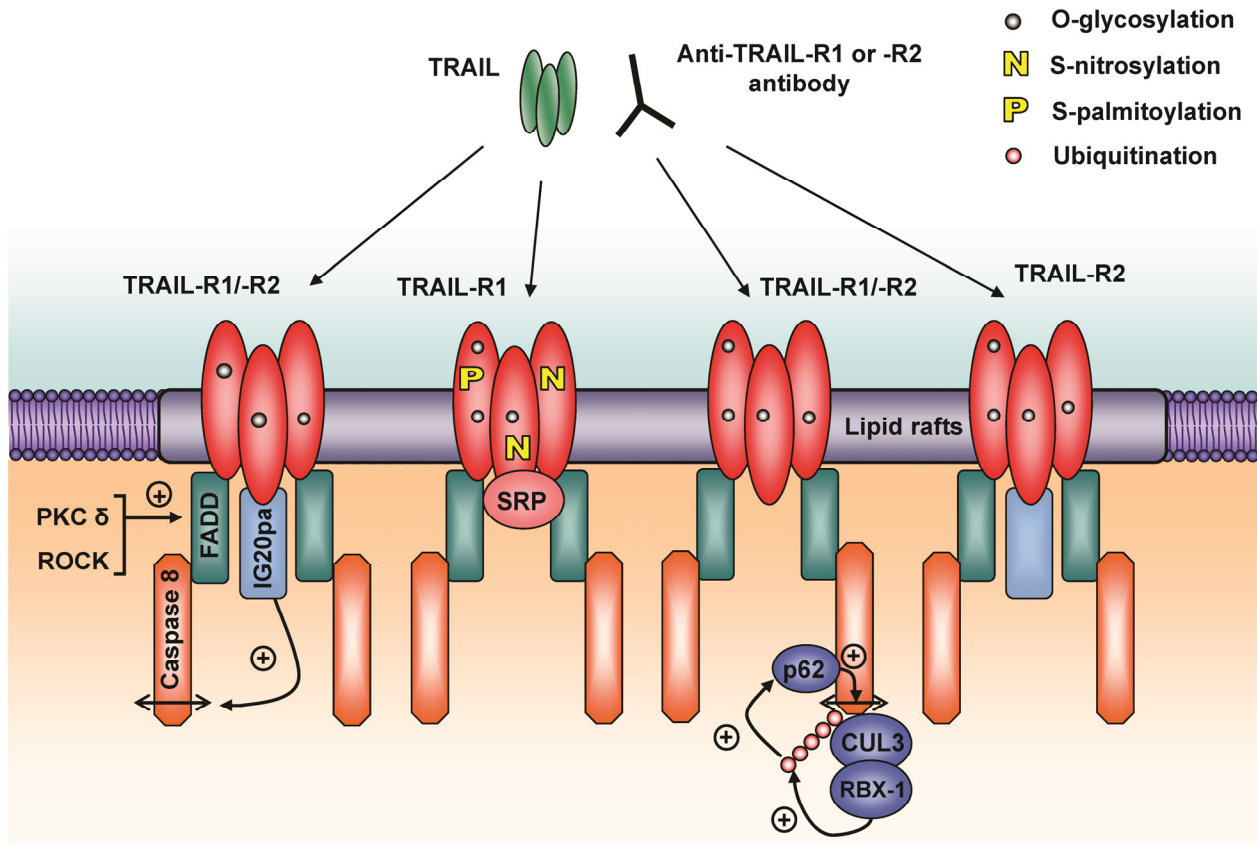


Figure 2: The “good” DISC. Several events contribute to optimal caspase 8 cleavage at the DISC. In TRAIL-sensitive cells, TRAIL receptor-specific agonists bind to their cognate TRAIL receptor trimers, inducing their aggregation in lipid rafts. In this figure, TRAIL-R1 and TRAIL-R2 specifically designate homotrimers, while TRAIL-R1/-R2 refers to either type of trimer, including possible heterotrimers. Modified forms of the TRAIL death receptors such as O-glycosylated TRAIL death receptors, S-nitrosylated TRAIL-R1 or S-palmitoylated TRAIL-R1 are more prone to aggregation and initiation of DISC formation. The SRP protein complex is involved in stabilizing TRAIL-R1 at the cell membrane, which increases its sensitivity to TRAIL-R1-specific agonists. Upon TRAIL death receptor stimulation, FADD is recruited to the TRAIL death receptor trimers, leading to caspase 8 recruitment and its cleavage into an active p18 cytoplasmic fragment. DISC formation and stability can be increased by IG20pa binding, PKCδ- or ROCK-dependent signals, thereby increasing caspase 8 processing. Caspase 8 cleavage is also enhanced by CUL3- and RBX-1-dependent polyubiquitination of caspase 8, which allows p62 binding, triggering both caspase 8 aggregation and auto-proteolytic cleavage of this protein.

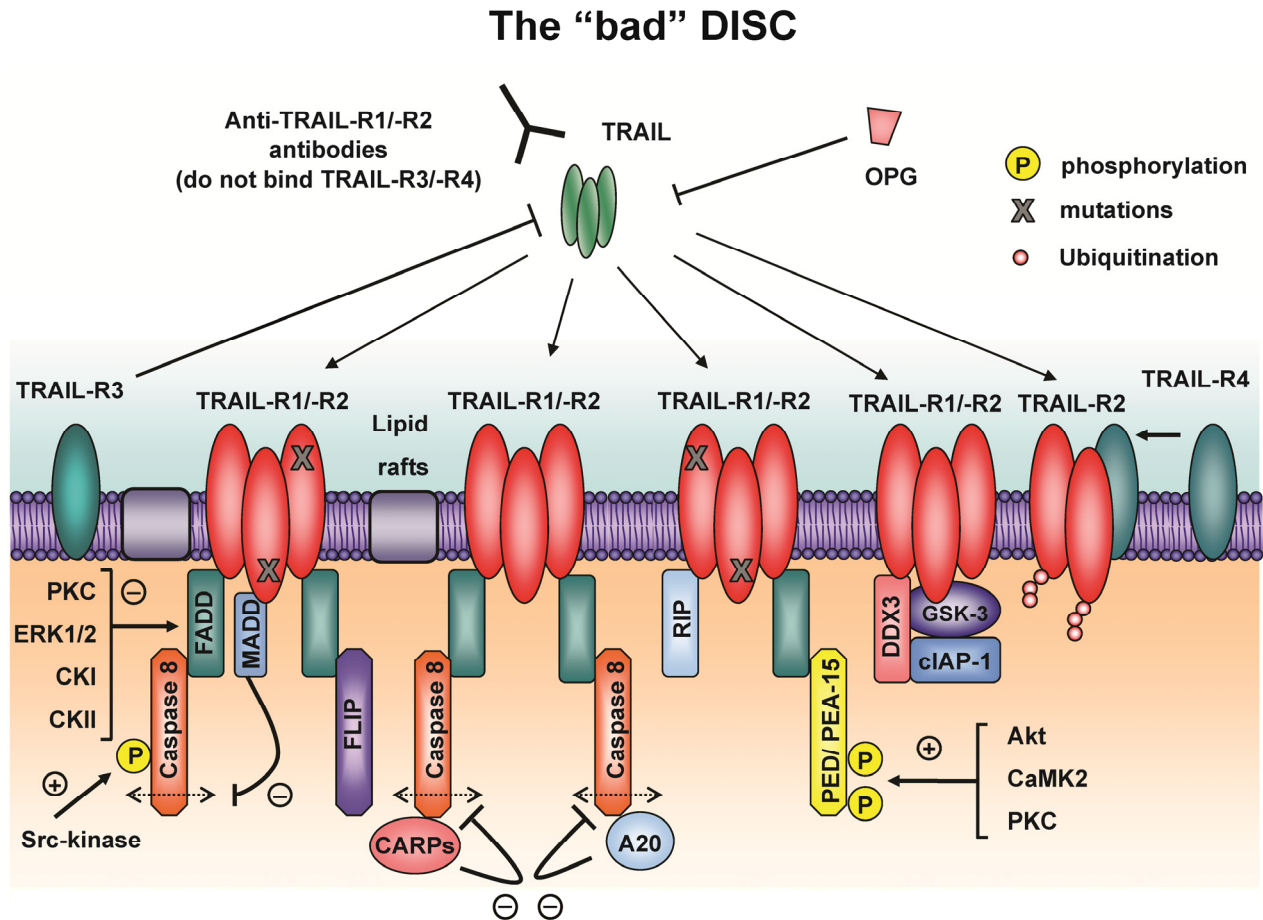


Figure 3: The “bad” DISC. Efficient DISC formation can be inhibited by numerous mechanisms in TRAIL-resistant cells. The soluble receptor to TRAIL (OPG) or the membrane TRAIL-R3/-R4 can prevent TRAIL binding to the TRAIL death receptors and/ or formation of pro-apoptotic membrane complexes (“Good” DISCs). TRAIL-R4 notably forms heterotrimer with TRAIL-R2. TRAIL-R2 can be directly ubiquitinated and targeted to the proteasome for degradation. Some mutations found in the TRAIL death receptors hamper efficient TRAIL binding or FADD recruitment. DISC formation is less efficient in absence of TRAIL death receptor recruitment to the lipid rafts. If, however, DISC formation is to take place outside of the lipid rafts, non-functional complexes (“Bad” DISCs) are formed and stimulate pro-survival pathways. FADD recruitment to the TRAIL death receptor can be inhibited by PKC-, ERK1/2- and CK-dependent signals. Several proteins and proteins complexes can be recruited to the DISC, preventing caspase 8 cleavage and/ or stimulating pro-survival pathways. The list of such proteins and protein complexes includes FLIP, RIP, PED/PEA-15 and the TRAIL death receptor-capping DDX3/cIAP-1/GSK-3 complex. Recruitment to FADD of PED/PEA-15 is positively regulated by its double-phosphorylation, under the influence of Akt, CAMK2 and PKC signaling. MADD binds to the TRAIL death receptor and prevents auto-proteolytic cleavage of caspase 8. Phosphorylation of caspase 8 by Src kinase also prevents its cleavage at the DISC. CARPs, namely CARP1 and CARP2, are ubiquitin-ligase proteins that promote caspase 8/10 ubiquitination and block proteolytic processing of these caspases. Conversely, A20 binds caspase 8, thereby preventing its pro-apoptotic polyubiquitination by CUL3 and caspase 8 auto-proteolytic processing.

donor nitrosylcobalamin (NO-Cbl), a pro-drug based on vitamin B12 (see Figure 2). Site-directed mutagenesis identified the amino-acid 336 as the S-nitrosylated cysteine residue in TRAIL-R1. Proliferation assays established that the TRAIL-R1 C336A mutant was less sensitive to NO-Cbl or TRAIL than other point mutants or vector-transfected cells, and showed reduced caspase 8 activation following NO-Cbl treatment. Since endogenous TRAIL-R1 S-nitrosylation was not detected in any of the three types of cancer cell lines tested, further experiments are needed to evaluate the extent of these findings in both normal and cancer cells (196).

3. S-palmitoylation of TRAIL-R1 promotes its oligomerization

The reversible attachment of a palmitate to a cysteine residue, a process also known as S-palmitoylation, can induce lipid raft localization of membrane receptors (197). Rossin *et al.* showed that TRAIL-R1 was partially palmitoylated within a cysteine triplet near its transmembrane domain (see Figure 2). Palmitoylation of TRAIL-R1 was required for its constitutive lipid raft localization and homo-oligomerization. In an overexpression system, only palmitoylated TRAIL-R1 could hetero-oligomerize with TRAIL-R2. TRAIL-R2 itself lacks the cystein triplet and accordingly was not found to be palmitoylated. Pre-treating cells with the palmitate analog 13-oxypalmitate or the cholesterol-depleting agent cholesterol oxydase hampered TRAIL-R1 constitutive lipid raft localization, decreased TRAIL-induced DISC formation and subsequently inhibited apoptotic cell death. Stimulation of the intrinsic apoptotic pathway with staurosporine in combination with cholesterol oxydase did not induce cell death. This suggests that cholesterol oxydase specifically affects the extrinsic pathway of apoptosis. Site-directed mutagenesis from cysteine to serine confirmed the C261-3S cysteine triplet to be critical to TRAIL-induced caspase 8 cleavage (198).

4. Caspase 8 phosphorylation prevents its cleavage at the DISC

Caspase 8 activity can be inhibited by phosphorylation of its tyrosine-380 by Src kinase (see Figure 3). TRAIL-resistant DLD1 cells transfected with a plasmid coding for a non-phosphorylatable caspase 8 mutant were more sensitive to TRAIL than their counterparts transfected with the wt caspase 8 gene. In support of these findings, the Src inhibitor PP2 increased TRAIL-induced caspase 8 cleavage and apoptosis in several cell lines, independent of TRAIL death receptor upregulation (199). Importantly, the

combination of TRAIL and PP2 was not toxic toward primary hepatocytes (200). Src levels are high in lipid rafts and these rafts have been shown to influence TRAIL death receptor aggregation and DISC formation. It remains to be tested whether Src kinase-dependent caspase 8 phosphorylation is increased in lipid rafts.

5. FLIP phosphorylation lowers its affinity for the DISC

In addition to caspase 8 phosphorylation, FLIP phosphorylation has also been described. The bile acid GCDCA did not affect protein expression of DISC components in hepatocellular cancer cells. However, pre-treatment with GCDCA increased caspase 8 and 10 recruitment at the TRAIL-DISC by phosphorylating both FLIP_L and the short isoform (FLIP_s), thereby lowering their affinity for FADD (see Figure 3). These effects could be prevented by the PKC inhibitor chelerythrine, suggesting an involvement of this pathway in regulating phosphorylation of the FLIP isoforms and their translocation to the TRAIL-DISC (201). Surprisingly, the authors did not discuss whether the increase in DISC formation could also be attributed to the higher clustering of TRAIL-R2 seen upon GCDCA treatment as reported in one of their earlier publications (170,201).

6. DISC protein ubiquitination differentially regulates DISC composition

TRAIL-R2 can be ubiquitinated, and its stability increased, by the proteasome inhibitor bortezomib, leading to higher TRAIL sensitivity (202)(see Figure 2). Although both FLIP isoforms can be ubiquitinated, FLIP_s was shown to be more prone to ubiquitination than FLIP_L and consequently exhibits a shorter half-life (203). Peroxisome-activated receptor γ (PPAR- γ) ligands and 2,5-dimethyl-celecoxib, a derivative of the COX-2 inhibitor celecoxib without COX-2 inhibitory activity, triggered ubiquitination of FLIP, induced degradation of both isoforms and sensitized various tumor cells to TRAIL (204,205). The effects of the PPAR- γ ligands seemed PPAR- γ -independent (205). In a yeast two-hybrid screen, two caspase 8- and 10-associated RING proteins (CARPs) with E3 ubiquitin ligase activity were identified by McDonald and El-Deiry (206). CARP1 and CARP2 interact with caspase 8 and 10 prodomains via their RING domain, which promotes their ubiquitination and subsequent proteasomal degradation. Downregulation of CARP1 and CARP2 sensitized colon and lung cancer cells to TRAIL-induced apoptosis by increasing cleavage of caspase 8/10. It is noteworthy that CARP downregulation did not modulate the basal expression levels of caspase 8/10. CARP1 and CARP2 were

upregulated in various cancer cell lines, and in a number of primary tumors compared to normal tissue. In a recent publication, Jin *et al.* uncovered a novel mechanism of caspase 8 (but not caspase 10) polyubiquitination, with opposite consequences for apoptosis initiation (207). In TRAIL-sensitive cells of various origins, TRAIL death receptor stimulation leads to DISC formation and translocation into lipid rafts, where a cullin-based E3 ligase (CUL3) interacted with caspase 8 and induced a ring-box 1 (RBX1)-dependent polyubiquitination of caspase 8. The presence of polyubiquitin in the C-terminal region of caspase 8 triggered recruitment of the ubiquitin-binding protein p62, which targeted DISC caspase 8 to ubiquitin-rich foci. Aggregation of caspase 8 in these high molecular weight structures promoted auto-proteolytic cleavage and the release of active caspase 8 into the cytoplasm, leading to executioner caspases activation and ultimately apoptosis. Using tandem mass spectrometry on purified high molecular weight DISC fractions, the authors also identified a caspase 8-bound deubiquitinase named A20, overexpression of which could prevent CUL3-induced caspase 8 ubiquitination and caspase 8 activity (see Figure 2). Caspase 8 ubiquitination thus appears to be a tightly regulated process involving several non-canonical DISC proteins. Downstream of the DISC in the TRAIL apoptotic pathway, Bcl-2 family proteins and executioner caspases are also regulated by ubiquitination (208,209). Altogether, changes in the balance between pro- and anti-apoptotic factors may account for the potent effects of proteasome inhibition on DISC formation and TRAIL sensitivity.

L. Influence of survival pathways on DISC formation

Beyond their role in cell cycle regulation, metabolism and cell differentiation, survival pathways can modulate TRAIL death receptor-mediated apoptosis. Studies show that numerous survival pathways are stimulated by the TRAIL apoptotic pathway, potentially initiating feedback loop mechanisms (36,210). It is now clear that survival pathways can also directly influence the apical events of DISC formation (Figure 2 and 3). Since these signals are primarily mediated by kinases, the effects can occur without changing the cytoplasmic protein levels of DISC proteins (211-213). The frequent enrichment of kinases at the membrane, particularly within lipid rafts, supports the possibility that close proximity might facilitate modulation of TRAIL death receptor-mediated signaling by these kinases.

1. The pro- and anti-DISC effects of protein kinase C

Protein kinase C (PKC) describes a group of 12 lipid-dependent serine/threonine (S/T) kinases that regulate growth and differentiation. Stimulating this pathway with the PKC activator phorbol 12,13-dibutyrate (PDBu) inhibited caspase 8 cleavage in response to TRAIL treatment in Jurkat cells. This decrease in TRAIL sensitivity was partly due to PKC-induced activation of the MAPK pathway (214). Meng *et al.* further demonstrated that caspase 8 cleavage was inhibited following treatment with the PKC activator phorbol 12-myristate 13-acetate (PMA) in type II cell lines from different tumor types. This was caused by disruption of FADD recruitment at the DISC (215). Similar results were obtained in the cervical cancer cell line Hela, where PKC stimulation using PMA could prevent FADD recruitment to the TRAIL death receptors (216)(see Figure 3). This strongly indicates that PKC can exert inhibitory effects on DISC formation in several cell types. The unique phosphorylation site of FADD (Serine-194) has been shown to regulate its non-apoptotic functions (217). It is therefore unlikely that the increase in FADD recruitment at the DISC seen upon PKC stimulation is caused by a PKC-dependent phosphorylation of FADD itself.

Studies specifically focusing on the δ isoform of PKC have led to opposite discoveries regarding the regulation of DISC assembly by PKC. Sandra *et al.* showed that the δ isoform of PKC could accelerate caspase 8 cleavage at the DISC following a positive feedback loop possibly involving the mitochondria. This feedback loop was initiated by a rapid production of diacylglycerol following TRAIL treatment, which induced activation of PKC δ and its relocation to the mitochondria, in a caspase-independent manner (218). Although the precise mechanism between this activation of PKC δ and the increase in caspase 8 cleavage is unknown, a Rho kinase (ROCK)-dependent contraction of myosin light chains has been shown to promote initiator caspases binding to FADD following PKC activation (219) (see Figure 2). A better knowledge of the properties of the different isoforms would facilitate the development of specific PKC inhibitors which would not inhibit both the anti- and pro-apoptotic forms.

2. *The ERK1/2 signaling pathway inhibits caspase 8 cleavage*

The ERK1/2 or MAPK pathway is an important cell survival pathway involved in carcinogenesis. This pathway has also been shown to inhibit TRAIL sensitivity at the DISC (see Figure 3). Frankel *et al.* found that the cytoplasmic domain of TRAIL-R1 but not TRAIL-R2 was constitutively phosphorylated by p42^{MAPK/ERK2} in unstimulated peripheral blood lymphocytes (220). This suggests the possibility for a direct regulation of TRAIL-R1 activity by the ERK pathway. In activated T-cells, the MAPK/ERK signaling abrogated TRAIL-induced apoptosis upstream of caspase 8 and the mitochondrial amplification feedback loop (221). Similarly, MAPK kinase 1 (MEKK1) inhibition in Hela cells enhanced TRAIL-induced apoptosis while expression of a constitutively active (CA) MEKK1 could protect cells from TRAIL death receptor-mediated apoptosis. TRAIL-induced caspase 8 processing was inhibited in cells expressing CA-MEKK1. Interestingly, TRAIL death receptor stimulation induced ERK1/2 activation, which appeared 5 min after treatment and lasted for about 1 h, thereby suggesting the involvement of this pathway in a negative feedback loop at the DISC (222).

3. *The anti-DISC effects of active CK1 α and CK2 α*

Casein kinase I α isoform (CK1 α) and protein kinase CK2 α , formerly known as casein kinase 2 (CK2 α), are serine/threonine kinases which can modulate DISC formation (see Figure 2). In colon cancer and pediatric rhabdomyosarcoma cell lines, TRAIL sensitivity dramatically increased upon inhibition of CKI with CKI-7 or of CK2 with 5,6-dichlorobenizimidazole (DRB). Formation and stability of the TRAIL-DISC were increased following CKI inhibition, while CK2 inhibition with DRB increased both TRAIL-induced caspase 8 recruitment at the DISC and TRAIL-induced apoptosis (223-225). Caspase 8 cleavage can be enhanced following feedback activation from executioner caspases downstream of the mitochondria such as caspase 6 (226). Blockade of the mitochondrial amplification pathway using Bcl-2 or Bcl-xL overexpression did not hamper caspase 8 cleavage in presence of TRAIL together with CKI or CK2 inhibitors. This confirms that CKs can modulate caspase 8 cleavage at the DISC itself. Active casein kinases such as CKI might inhibit TRAIL-induced apoptosis by phosphorylating the TRAIL death receptor, thereby blocking FADD recruitment at the DISC (223-225).

M. DISC regulation by recruitment of additional proteins

Beyond the DISC components discussed earlier in this review, several other non-canonical proteins have been found to interact with the DISC at the membrane, where they either positively or negatively influence efficient DISC formation, caspase 8 cleavage and consequent apoptosis. A schematic representation of the recruitment of these pro- and anti-apoptotic proteins to the DISC is given in Figure 2 and 3, respectively and the mechanisms are discussed more extensively below.

1. *PED/PEA-15 blocks caspase 8 cleavage at the DISC*

Phosphoprotein enriched in diabetes/ phosphoprotein enriched in astrocytoma-15 kDa (PED/ PEA-15) belongs to the category of non-canonical DISC proteins. Like FADD, FLIP and caspase 8, PED/PEA-15 possesses a death effector domain but no caspase-like domain. Hao *et al.* showed that PED/PEA-15 expression was about two times higher in TRAIL-resistant compared to TRAIL-sensitive glioma cell lines (227). PED/PEA-15 was not found in the TRAIL-DISC of three TRAIL-resistant primary gliomas (77). Overexpression of PED/PEA-15 in TRAIL-sensitive glioma cell lines blocked the TRAIL apoptotic pathway while a PED/PEA-15 anti-sense approach restored TRAIL sensitivity in TRAIL-resistant gliomas (227). PED/PEA-15 exists in three isoforms, namely an unphosphorylated, a singly-phosphorylated and a doubly-phosphorylated form. Calcium/calmodulin-dependent protein kinase II (CaMK2) or Akt phosphorylate PED/PEA-15 at serine-116 (228-230), thereby facilitating the second phosphorylation at serine-104 by PKC (231,232). Only the double phosphorylated form, present exclusively in TRAIL-resistant glioma cells, was found to be recruited to the TRAIL-DISC where it blocked caspase 8 activation (230)(see Figure 3). Inhibition of CaMK2 with the broad-spectrum CaMK inhibitor KN-93 decreased PED/PEA-15 phosphorylation, thereby restoring caspase 8 cleavage and apoptosis in gliomas (230). Inhibition of Akt or PKC also hindered PED/PEA-15 phosphorylation, its recruitment at the DISC and restored TRAIL sensitivity in glioma cell lines (227,229). These results are consistent with the role of PED/PEA-15 as a common target for multiple survival pathways, allowing these pathways to modulate TRAIL-induced apoptosis directly at the DISC.

2. Blockade of DISC formation by the DDX3/GSK3/cIAP-1 capping complex

Li *et al.* described a novel protein specifically involved in acquired resistance to TRA-8, an agonistic anti-TRAIL-R2 antibody. DDX3, a member of the DEAD-box RNA helicase family, was endogenously associated with TRAIL-R2. Upon stimulation of the TRAIL-sensitive breast cancer cell line MDA-MB-231 with TRA-8, DDX3 was cleaved and an active DISC was formed. In the TRA-8-resistant daughter cell line, the TRA-8 antibody failed to induce cleavage of DDX3. FADD and caspase 8 were not recruited to TRAIL-R2, suggesting a role for DDX3 in inhibiting this process. Resistance to the anti-TRAIL-R2 antibody could be reversed by several chemotherapeutic agents, including doxorubicin, which restored FADD recruitment and cleavage of both DDX3 and caspase 8 (233). DDX3, together with GSK3 and cIAP-1, formed a capping complex at the DISC that counteracted TRAIL death receptor-mediated apoptosis (see Figure 3). Additionally, DDX3 and cIAP-1 were also recruited to the DISC in both sensitive and resistant cells following TRAIL death receptor stimulation but cleavage and inactivation of this capping complex was only induced in TRAIL-sensitive cells. In various cell lines, inhibition of GSK3 with compounds such as lithium was found to promote TRAIL death receptor-mediated apoptosis by increasing DISC formation (234). Altogether, these findings are particularly relevant in view of the numerous GSK3 inhibitors and IAP small molecule antagonists that are currently in clinical development (209,235).

3. The pro- and anti-DISC properties of the various IG20 gene family isoforms

The insulinoma-glucagonoma clone 20 (IG20) gene is frequently overexpressed in cancer and encodes several splice variants with various effects on cell proliferation and drug sensitivity (236). The IG20 pro-apoptotic (IG20pa) splice variant was shown to directly interact with both TRAIL-R1 and TRAIL-R2 (237) but not with FADD (238)(see Figure 2). Cancer cells transfected with IG20pa displayed a higher recruitment of FADD and caspase 8 to TRAIL-R1 and TRAIL-R2 in response to TRAIL treatment, and were also more sensitive to this ligand (237,239). The precise mechanism behind this DISC-enhancing effect is unknown, but IG20pa is most likely recruited to the TRAIL death receptors via its C-terminal 70 amino-acid death domain homology region (237).

Unlike the IG20pa variant, the MAPK-activating death domain protein (MADD) splice variant of IG20 is a negative regulator of caspase 8 activation at the DISC (see Figure 3). Downregulation of endogenous MADD alone resulted in spontaneous

apoptosis via ligand-independent caspase 8 cleavage at the DISC, and also increased TRAIL sensitivity. MADD binds to the TRAIL death receptors but not to FADD or caspase 8 itself, and although it does not prevent DISC assembly, MADD inhibits caspase 8 cleavage at the DISC (240,241). In Kaposi's sarcoma cells, MADD could be downregulated with the antineoplastic drug and TRAIL sensitizer actinomycin D (242,243). These results further confirm that versatile mechanisms regulate TRAIL sensitivity at the DISC itself.

4. Regulation of cell membrane TRAIL-R1 levels by the signal recognition particle complex

In search for proteins differentially regulating TRAIL-R1- and TRAIL-R2-mediated apoptosis in cancer cells, Ren *et al.* tested an arrayed library of siRNAs in combination with TRAIL-R1- or TRAIL-R2-specific antibodies, in HCT15 colon cancer cells (244). Several proteins were identified as pro- and anti-apoptotic for TRAIL-R1- and/or TRAIL-R2-mediated signaling. The authors found that expression of the signal recognition particle (SRP) complex was essential for TRAIL-R1-mediated sensitivity (see Figure 2). Downregulation of the 54-kDa or 72-kDa (SRP54 or SRP72) subunits of the SRP complex decreased cell sensitivity to TRAIL and to anti-TRAIL-R1 antibody, but not to anti-TRAIL-R2 antibody. Effects of SRP54 or SRP72 siRNAs on TRAIL sensitivity were also seen in cervical and pancreatic cell lines. The SRP complex is notably involved in the sorting process of nascent membrane proteins (245,246), which can be linked to the finding that cell membrane TRAIL-R1 levels decreased upon inhibition of SRP54 or SRP72 expression (244). In addition to the studies on TRAIL-R1 epigenetic silencing and endocytosis mentioned earlier in this review, these results support the hypothesis that cell membrane TRAIL-R1 downregulation contributes to TRAIL resistance.

N. Conclusions

So far, much focus has been placed on finding ways to modulate pro- and anti-apoptotic protein expression to enhance TRAIL-induced apoptosis. The efficacy of such combinations *in vitro* and in xenograft models is unquestionable, and preliminary clinical data are encouraging. However, to fully exploit the apoptotic effect of any such combination treatment, there should be more insight in the mechanisms regulating

TRAIL sensitization, DISC formation and caspase 8 activation. In view of the vast range of potential targets offered and rapidly accumulating data, the use of post-translational approaches to regulate the TRAIL-induced apoptotic cascade is also suggested. Furthermore, although TRAIL-R1 and TRAIL-R2 agonists both transduce apoptosis, extensive data suggests that TRAIL-R1- and TRAIL-R2-mediated signaling cascades are fine-tuned by different mechanisms. Even more so than TRAIL, receptor-selective agonists such as anti-TRAIL-R1 or anti-TRAIL-R2 antibodies or receptor-specific TRAIL variants are targeted therapies that necessitate screening for the presence of functional TRAIL death receptor in addition to screening for other DISC components. Elucidating potential TRAIL-R1- or TRAIL-R2-specific effects of novel and existing chemotherapies will be crucial to fully exploit the clinical potential of these TRAIL death receptor-selective agonists. In the meantime, the best strategy may be to combine these chemotherapies with dual targeting of both TRAIL-R1 and TRAIL-R2, using either TRAIL or a combination of TRAIL-R1- plus TRAIL-R2-selective agonists.

Acknowledgements

Supported by grants RUG 2005-3361, RUG 2005-3365 and VU 2006-3567 from the Dutch Cancer Society, the EU FP6 program LSHC-CT-2006-037686 and TI Pharma T3.112

References

1. Hengartner M. The biochemistry of apoptosis. *Nature* 2000; 407: 770-776.
2. Kerr JF, Wyllie AH and Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; 26: 239-257.
3. Danial N and Korsmeyer S. Cell death: critical control points. *Cell* 2004; 116: 205-219.
4. Reed J and Tomaselli K. Drug discovery opportunities from apoptosis research. *Curr Opin Biotechnol* 2000; 11: 586-592.
5. Takano Y, Saegusa M, Ikenaga M, Mitomi H and Okayasu I. Apoptosis of colon cancer: Comparison with Ki-67 proliferative activity and expression of p53. *J Cancer Res Clin Oncol* 1996; 122: 166-170.
6. Koornstra JJ, de Jong S, Hollema H, de Vries EGE and Kleibeuker JH. Changes in apoptosis during the development of colorectal cancer: a systematic review of the literature. *Crit Rev Oncol Hematol* 2003; 45: 37-53.
7. Koornstra JJ, Rijcken FE, de Jong S, Hollema H, de Vries EGE and Kleibeuker JH. Assessment of apoptosis by M30 immunoreactivity and the correlation with morphological criteria in normal colorectal mucosa, adenomas and carcinomas. *Histopathology* 2004; 44: 9-17.
8. Fesik S. Promoting apoptosis as a strategy for cancer drug discovery. *Nat Rev Cancer* 2005; 5: 876-885.
9. Ashkenazi A and Dixit VM. Death receptors: signaling and modulation. *Science* 1998; 281: 1305-1308.
10. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA and . Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995; 3: 673-682.
11. van Geelen CMM, de Vries EGE, Le TKP, van Weeghel RP and de Jong S. Differential modulation of the TRAIL receptors and the CD95 receptor in colon carcinoma cell lines. *Br J Cancer* 2003; 89: 363-373.
12. Velthuis J, Rouschop K, de Bont H, Mulder G and Nagelkerke JF. Distinct Intracellular Signaling in Tumor Necrosis Factor-related Apoptosis-inducing Ligand- and CD95 Ligand-mediated Apoptosis. *J Biol Chem* 2002; 277: 24631-24637.
13. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T and Nagata S. Lethal effect of the anti-Fas antibody in mice. *Nature* 1993; 364: 806-809.
14. Oldenhuis CNAM, Stegehuis JH, Walenkamp AME, de Jong S and de Vries EGE. Targeting TRAIL death receptors. *Curr Opin Pharmacol* 2008; 8: 433-439.
15. Krammer PH, Arnold R and Lavrik IN. Life and death in peripheral T cells. *Nat Rev Immunol* 2007; 7: 532-542.

16. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A and Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996; 271: 12687-12690.
17. Duiker EW, Mom CH, de Jong S, Willemse PH, Gietema JA, van der Zee AG and de Vries EGE. The clinical trial of TRAIL. *Eur J Cancer* 2006; 42: 2233-2240.
18. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ and Smyth MJ. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol* 2002; 168: 1356-1361.
19. Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Kayagaki N, Yagita H and Okumura K. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. *J Exp Med* 2002; 195: 161-169.
20. Grosse-Wilde A, Voloshanenkov O, Bailey SL, Longton GM, Schaefer U, Csernok AI, Schutz G, Greiner EF, Kemp CJ and Walczak H. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. *J Clin Invest* 2008; 118: 100-110.
21. Hymowitz SG, O'Connell MP, Ultsch MH, Hurst A, Totpal K, Ashkenazi A, de Vos AM and Kelley RF. A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. *Biochemistry* 2000; 39: 633-640.
22. Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR and Strom SC. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med* 2000; 6: 564-567.
23. Nitsch R, Bechmann I, Deisz RA, Haas D, Lehmann TN, Wendling U and Zipp F. Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). *Lancet* 2000; 356: 827-828.
24. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z and Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999; 104: 155-162.
25. Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B, Hillan K, Totpal K, DeForge L, Schow P, Hooley J, Sherwood S, Pai R, Leung S, Khan L, Gliniak B, Bussiere J, Smith CA, Strom SS, Kelley S, Fox JA, Thomas D and Ashkenazi A. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* 2001; 7: 383-385.
26. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC and Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* 1999; 5: 157-163.
27. Herbst RS, Mendolson DS, Ebbinghaus S, Gordon MS, O'Dwyer P, Lieberman G, Ing J, Kurzrock R, Novotny W and Eckhardt G. A phase I safety and pharmacokinetic (PK) study of recombinant Apo2L/TRAIL, an apoptosis-inducing protein in patients with advanced cancer. *J Clin Oncol* 2006; 24: 124S.

28. Wang TT and Jeng J. Coordinated regulation of two TRAIL-R2/KILLER/DR5 mRNA isoforms by DNA damaging agents, serum and 17beta-estradiol in human breast cancer cells. *Breast Cancer Res Treat* 2000; 61: 87-96.
29. Degli-Esposti MA, Smolak PJ, Walczak H, Waugh J, Huang CP, DuBose RF, Goodwin RG and Smith CA. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med* 1997; 186: 1165-1170.
30. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA and Goodwin RG. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* 1997; 7: 813-820.
31. Kimberley FC and Screaton GR. Following a TRAIL: update on a ligand and its five receptors. *Cell Res* 2004; 14: 359-372.
32. Merino D, Lalaoui N, Morizot A, Schneider P, Solary E and Micheau O. Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2. *Mol Cell Biol* 2006; 26: 7046-7055.
33. Walczak H and Sprick MR. Biochemistry and function of the DISC. *Trends Biochem Sci* 2001; 26: 452-453.
34. Krueger A, Baumann S, Krammer PH and Kirchhoff S. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol Cell Biol* 2001; 21: 8247-8254.
35. Sprick MR, Rieser E, Stahl H, Grosse-Wilde A, Weigand MA and Walczak H. Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *EMBO J* 2002; 21: 4520-4530.
36. Falschlehner C, Emmerich CH, Gerlach B and Walczak H. TRAIL signalling: decisions between life and death. *Int J Biochem Cell Biol* 2007; 39: 1462-1475.
37. Mahalingam D, Szegezdi E, Keane M, de Jong S and Samali A. TRAIL receptor signalling and modulation: Are we on the right TRAIL? *Cancer Treat Rev* 2009; 35: 280-288.
38. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, Lee D, von Goetz M, Yee SF, Totpal K, Huw L, Katta V, Cavet G, Hymowitz SG, Amler L and Ashkenazi A. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. *Nat Med* 2007; 13: 1070-1077.
39. Johnstone RW. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat Rev Drug Discov* 2002; 1: 287-299.
40. van Geelen CMM, de Vries EGE and de Jong S. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist Updat* 2004; 7: 345-358.
41. Yang JK. FLIP as an anti-cancer therapeutic target. *Yonsei Med J* 2008; 49: 19-27.

42. Fanale M, Burris H, Yee L, Lucas J, Dimick K, Goldwasser M, Novotny W and Bray G. Results of a phase 1B study of recombinant human APO2L/trail with rituximab in patients with relapsed, low-grade NHL. *Ann Oncol* 2008; 19: 161.
43. Soria J, Smit E, Khayat D, Besse B, Burton J, Yang X, Hsu M, Braun A, Reese D and Blackhall FH. Safety, Pharmacokinetics, and Efficacy of Recombinant Human (Rh)Apo2L/Trail in Combination with Paclitaxel, Carboplatin, and Bevacizumab (Pcb) in Patients (Pts) with Advanced Non-Small Cell Lung Cancer (Nslc): Results of A Phase 1B Study. *Ann Oncol* 2008; 19: 153.
44. Hougardy BM, Maduro JH, van der Zee AG, de Groot DJ, van den Heuvel FA, de Vries EGE and de Jong S. Proteasome inhibitor MG132 sensitizes HPV-positive human cervical cancer cells to rhTRAIL-induced apoptosis. *Int J Cancer* 2006; 118: 1892-1900.
45. Yoshida T, Shiraishi T, Nakata S, Horinaka M, Wakada M, Mizutani Y, Miki T and Sakai T. Proteasome inhibitor MG132 induces death receptor 5 through CCAAT/enhancer-binding protein homologous protein. *Cancer Res* 2005; 65: 5662-5667.
46. Liu X, Yue P, Chen S, Hu L, Lonial S, Khuri FR and Sun SY. The proteasome inhibitor PS-341 (bortezomib) up-regulates DR5 expression leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. *Cancer Res* 2007; 67: 4981-4988.
47. Voortman J, Resende TP, bou El Hassan MA, Giaccone G and Kruyt FA. TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. *Mol Cancer Ther* 2007; 6: 2103-2112.
48. Koschny R, Ganten TM, Sykora J, Haas TL, Sprick MR, Kolb A, Stremmel W and Walczak H. TRAIL/bortezomib cotreatment is potentially hepatotoxic but induces cancer-specific apoptosis within a therapeutic window. *Hepatology* 2007; 45: 649-658.
49. Butler LM, Liapis V, Bouralexis S, Welldon K, Hay S, Thai IM, Labrinidis A, Tilley WD, Findlay DM and Evdokiou A. The histone deacetylase inhibitor, suberoylanilide hydroxamic acid, overcomes resistance of human breast cancer cells to Apo2L/TRAIL. *Int J Cancer* 2006; 119: 944-954.
50. Nakata S, Yoshida T, Horinaka M, Shiraishi T, Wakada M and Sakai T. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. *Oncogene* 2004; 23: 6261-6271.
51. Fandy TE, Shankar S, Ross DD, Sausville E and Srivastava RK. Interactive effects of HDAC inhibitors and TRAIL on apoptosis are associated with changes in mitochondrial functions and expressions of cell cycle regulatory genes in multiple myeloma. *Neoplasia* 2005; 7: 646-657.
52. Singh TR, Shankar S and Srivastava RK. HDAC inhibitors enhance the apoptosis-inducing potential of TRAIL in breast carcinoma. *Oncogene* 2005; 24: 4609-4623.
53. Earel JK, Jr., VanOosten RL and Griffith TS. Histone deacetylase inhibitors modulate the sensitivity of tumor necrosis factor-related apoptosis-inducing ligand-resistant bladder tumor cells. *Cancer Res* 2006; 66: 499-507.

54. VanOosten RL, Moore JM, Karacay B and Griffith TS. Histone deacetylase inhibitors modulate renal cell carcinoma sensitivity to TRAIL/Apo-2L-induced apoptosis by enhancing TRAIL-R2 expression. *Cancer Biol Ther* 2005; 4: 1104-1112.
55. Guo F, Sigua C, Tao J, Bali P, George P, Li Y, Wittmann S, Moscinski L, Atadja P and Bhalla K. Cotreatment with histone deacetylase inhibitor LAQ824 enhances Apo-2L/tumor necrosis factor-related apoptosis inducing ligand-induced death inducing signaling complex activity and apoptosis of human acute leukemia cells. *Cancer Res* 2004; 64: 2580-2589.
56. Inoue S, MacFarlane M, Harper N, Wheat LM, Dyer MJ and Cohen GM. Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. *Cell Death Differ* 2004; 11 Suppl 2: S193-S206.
57. Huang Y, He Q, Hillman MJ, Rong R and Sheikh MS. Sulindac sulfide-induced apoptosis involves death receptor 5 and the caspase 8-dependent pathway in human colon and prostate cancer cells. *Cancer Res* 2001; 61: 6918-6924.
58. Sinicrope FA and Penington RC. Sulindac sulfide-induced apoptosis is enhanced by a small-molecule Bcl-2 inhibitor and by TRAIL in human colon cancer cells overexpressing Bcl-2. *Mol Cancer Ther* 2005; 4: 1475-1483.
59. Liu X, Yue P, Zhou Z, Khuri FR and Sun SY. Death receptor regulation and celecoxib-induced apoptosis in human lung cancer cells. *J Natl Cancer Inst* 2004; 96: 1769-1780.
60. Shankar S, Singh TR and Srivastava RK. Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer in vitro and in vivo: Intracellular mechanisms. *Prostate* 2004; 61: 35-49.
61. Marini P, Schmid A, Jendrossek V, Faltin H, Daniel PT, Budach W and Belka C. Irradiation specifically sensitises solid tumour cell lines to TRAIL mediated apoptosis. *BMC Cancer* 2005; 5: 5.
62. Di PR, Secchiero P, Rana R, Gibellini D, Visani G, Bemis K, Zamai L, Miscia S and Zauli G. Ionizing radiation sensitizes erythroleukemic cells but not normal erythroblasts to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)--mediated cytotoxicity by selective up-regulation of TRAIL-R1. *Blood* 2001; 97: 2596-2603.
63. Maduro JH, de Vries EGE, Meersma GJ, Hougardy BM, van der Zee AG and de JS. Targeting pro-apoptotic trail receptors sensitizes HeLa cervical cancer cells to irradiation-induced apoptosis. *Int J Radiat Oncol Biol Phys* 2008; 72: 543-552.
64. Verbrugge I, de VE, Tait SW, Wissink EH, Walczak H, Verheij M and Borst J. Ionizing radiation modulates the TRAIL death-inducing signaling complex, allowing bypass of the mitochondrial apoptosis pathway. *Oncogene* 2008; 27: 574-584.
65. Dejosez M, Ramp U, Mahotka C, Krieg A, Walczak H, Gabbert HE and Gerharz CD. Sensitivity to TRAIL/APO-2L-mediated apoptosis in human renal cell carcinomas and its enhancement by topotecan. *Cell Death Differ* 2000; 7: 1127-1136.
66. LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicrope D and Ashkenazi A. Tumor-cell resistance to death receptor--induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nat Med* 2002; 8: 274-281.

67. Naka T, Sugamura K, Hylander BL, Widmer MB, Rustum YM and Repasky EA. Effects of tumor necrosis factor-related apoptosis-inducing ligand alone and in combination with chemotherapeutic agents on patients' colon tumors grown in SCID mice. *Cancer Res* 2002; 62: 5800-5806.
68. Wen J, Ramadevi N, Nguyen D, Perkins C, Worthington E and Bhalla K. Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of human acute leukemia cells. *Blood* 2000; 96: 3900-3906.
69. Shankar S, Chen X and Srivastava RK. Effects of sequential treatments with chemotherapeutic drugs followed by TRAIL on prostate cancer in vitro and in vivo. *Prostate* 2005; 62: 165-186.
70. Singh TR, Shankar S, Chen X, Asim M and Srivastava RK. Synergistic interactions of chemotherapeutic drugs and tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand on apoptosis and on regression of breast carcinoma in vivo. *Cancer Res* 2003; 63: 5390-5400.
71. Xiang H, Fox JA, Totpal K, Aikawa M, Dupree K, Sinicropi D, Lowe J and Escandon E. Enhanced tumor killing by Apo2L/TRAIL and CPT-11 co-treatment is associated with p21 cleavage and differential regulation of Apo2L/TRAIL ligand and its receptors. *Oncogene* 2002; 21: 3611-3619.
72. Mitsiades CS, Treon SP, Mitsiades N, Shima Y, Richardson P, Schlossman R, Hideshima T and Anderson KC. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. *Blood* 2001; 98: 795-804.
73. Wu XX, Takehi Y, Mizutani Y, Nishiyama H, Kamoto T, Megumi Y, Ito N and Ogawa O. Enhancement of TRAIL/Apo2L-mediated apoptosis by adriamycin through inducing DR4 and DR5 in renal cell carcinoma cells. *Int J Cancer* 2003; 104: 409-417.
74. Lacour S, Micheau O, Hammann A, Drouineaud V, Tschopp J, Solary E and manche-Boitrel MT. Chemotherapy enhances TNF-related apoptosis-inducing ligand DISC assembly in HT29 human colon cancer cells. *Oncogene* 2003; 22: 1807-1816.
75. Song JH, Tse MC, Bellail A, Phuphanich S, Khuri F, Kneteman NM and Hao C. Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and nonapoptotic signals in non small cell lung carcinoma cells. *Cancer Res* 2007; 67: 6946-6955.
76. Ganten TM, Haas TL, Sykora J, Stahl H, Sprick MR, Fas SC, Krueger A, Weigand MA, Grosse-Wilde A, Stremmel W, Krammer PH and Walczak H. Enhanced caspase-8 recruitment to and activation at the DISC is critical for sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by chemotherapeutic drugs. *Cell Death Differ* 2004; 11 Suppl 1: S86-S96.
77. Koschny R, Holland H, Sykora J, Haas TL, Sprick MR, Ganten TM, Krupp W, Bauer M, Ahnert P, Meixensberger J and Walczak H. Bortezomib sensitizes primary human astrocytoma cells of WHO grades I to IV for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Clin Cancer Res* 2007; 13: 3403-3412.
78. Kyritsis AP, Tachmazoglou F, Rao JS and Puduvalli VK. Bortezomib sensitizes human astrocytoma cells to tumor necrosis factor related apoptosis-inducing ligand induced apoptosis. *Clin Cancer Res* 2007; 13: 6540-6541.

79. Sohn D, Totzke G, Essmann F, Schulze-Osthoff K, Levkau B and Janicke RU. The proteasome is required for rapid initiation of death receptor-induced apoptosis. *Mol Cell Biol* 2006; 26: 1967-1978.
80. MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM and Alnemri ES. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J Biol Chem* 1997; 272: 25417-25420.
81. Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, Yuan J, Gurney A, Goddard AD, Godowski P and Ashkenazi A. A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr Biol* 1997; 7: 1003-1006.
82. Wu CL, Roz L, Sloan P, Read AP, Holland S, Porter S, Scully C, Speight PM and Thakker N. Deletion mapping defines three discrete areas of allelic imbalance on chromosome 8p in oral and oropharyngeal squamous cell carcinomas. *Genes Chrom Cancer* 1997; 20: 347-353.
83. el Naggar AK, Coombes MM, Batsakis JG, Hong WK, Goepfert H and Kagan J. Localization of chromosome 8p regions involved in early tumorigenesis of oral and laryngeal squamous carcinoma. *Oncogene* 1998; 16: 2983-2987.
84. Emi M, Fujiwara Y, Nakajima T, Tsuchiya E, Tsuda H, Hirohashi S, Maeda Y, Tsuruta K, Miyaki M and Nakamura Y. Frequent loss of heterozygosity for loci on chromosome 8p in hepatocellular carcinoma, colorectal cancer, and lung cancer. *Cancer Res* 1992; 52: 5368-5372.
85. Fujiwara Y, Ohata H, Emi M, Okui K, Koyama K, Tsuchiya E, Nakajima T, Monden M, Mori T, Kurimasa A and . A 3-Mb physical map of the chromosome region 8p21.3-p22, including a 600-kb region commonly deleted in human hepatocellular carcinoma, colorectal cancer, and non-small cell lung cancer. *Genes Chrom Cancer* 1994; 10: 7-14.
86. Kagan J, Stein J, Babaian RJ, Joe YS, Pisters LL, Glassman AB, von Eschenbach AC and Troncoso P. Homozygous deletions at 8p22 and 8p21 in prostate cancer implicate these regions as the sites for candidate tumor suppressor genes. *Oncogene* 1995; 11: 2121-2126.
87. MacGrogan D, Levy A, Bostwick D, Wagner M, Wells D and Bookstein R. Loss of chromosome arm 8p loci in prostate cancer: mapping by quantitative allelic imbalance. *Genes Chrom Cancer* 1994; 10: 151-159.
88. Mitelman F, Mertens F and Johansson B. A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. *Nat Genet* 1997; 15 Spec No: 417-474.
89. Monni O, Joensuu H, Franssila K and Knuutila S. DNA copy number changes in diffuse large B-cell lymphoma--comparative genomic hybridization study. *Blood* 1996; 87: 5269-5278.
90. Wistuba II, Behrens C, Virmani AK, Milchgrub S, Syed S, Lam S, Mackay B, Minna JD and Gazdar AF. Allelic losses at chromosome 8p21-23 are early and frequent events in the pathogenesis of lung cancer. *Cancer Res* 1999; 59: 1973-1979.
91. Wright K, Wilson PJ, Kerr J, Do K, Hurst T, Khoo SK, Ward B and Chenevix-Trench G. Frequent loss of heterozygosity and three critical regions on the short arm of chromosome 8 in ovarian adenocarcinomas. *Oncogene* 1998; 17: 1185-1188.

92. Yaremko ML, Recant WM and Westbrook CA. Loss of heterozygosity from the short arm of chromosome 8 is an early event in breast cancers. *Genes Chrom Cancer* 1995; 13: 186-191.
93. Bae SI, Cheriya V, Jacobs BS, Reu FJ and Borden EC. Reversal of methylation silencing of Apo2L//TRAIL receptor 1 (DR4) expression overcomes resistance of SK-MEL-3 and SK-MEL-28 melanoma cells to interferons (IFNs) or Apo2L//TRAIL. *Oncogene* 2007; 27: 490-498.
94. Horak P, Pils D, Haller G, Pribill I, Roessler M, Tomek S, Horvat R, Zeillinger R, Zielinski C and Krainer M. Contribution of epigenetic silencing of tumor necrosis factor-related apoptosis inducing ligand receptor 1 (DR4) to TRAIL resistance and ovarian cancer. *Mol Cancer Res* 2005; 3: 335-343.
95. Rubio-Moscardo F, Blesa D, Mestre C, Siebert R, Balasas T, Benito A, Rosenwald A, Climent J, Martinez JJ, Schilhabel M, Karran EL, Gesk S, Esteller M, deLeeuw R, Staudt LM, Fernandez-Luna JL, Pinkel D, Dyer MJ and Martinez-Climent JA. Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. *Blood* 2005; 106: 3214-3222.
96. Ozoren N, Fisher MJ, Kim K, Liu CX, Genin A, Shifman Y, Dicker DT, Spinner NB, Lisitsyn NA and El Deiry WS. Homozygous deletion of the death receptor DR4 gene in a nasopharyngeal cancer cell line is associated with TRAIL resistance. *Int J Oncol* 2000; 16: 917-925.
97. Duiker EW, de Vries EGE, Mahalingam D, Meersma GJ, Boersma-van EW, Hollema H, Lub-de Hooge MN, van Dam GM, Cool RH, Quax WJ, Samali A, van der Zee AG and de Jong S. Enhanced antitumor efficacy of a DR5-specific TRAIL variant over recombinant human TRAIL in a bioluminescent ovarian cancer xenograft model. *Clin Cancer Res* 2009; 15: 2048-2057.
98. Jin Z, McDonald ER, III, Dicker DT and El-Deiry WS. Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. *J Biol Chem* 2004; 279: 35829-35839.
99. Kim K, Fisher MJ, Xu SQ and El Deiry WS. Molecular determinants of response to TRAIL in killing of normal and cancer cells. *Clin Cancer Res* 2000; 6: 335-346.
100. Zhang Y and Zhang B. TRAIL Resistance of Breast Cancer Cells Is Associated with Constitutive Endocytosis of Death Receptors 4 and 5. *Mol Cancer Res* 2008; 6: 1861-1871.
101. Koornstra JJ, Kleibeuker JH, van Geelen CMM, Rijcken FE, Hollema H, de Vries EGE and de Jong S. Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonic mucosa, adenomas, and carcinomas. *J Pathol* 2003; 200: 327-335.
102. Arts HJ, de Jong S, Hollema H, ten Hoor K, van der Zee AG and de Vries EGE. Chemotherapy induces death receptor 5 in epithelial ovarian carcinoma. *Gynecol Oncol* 2004; 92: 794-800.
103. Imtiyaz H, Zhou X, Zhang H, Chen D, Hu T and Zhang J. The Death Domain of FADD Is Essential for Embryogenesis, Lymphocyte Development, and Proliferation. *J Biol Chem* 2009; 284: 9917-9926.
104. Garofalo M, Quintavalle C, Di LG, Zanca C, Romano G, Taccioli C, Liu CG, Croce CM and Condorelli G. MicroRNA signatures of TRAIL resistance in human non-small cell lung cancer. *Oncogene* 2008; 27: 3845-3855.

105. Ovcharenko D, Kelnar K, Johnson C, Leng N and Brown D. Genome-scale microRNA and small interfering RNA screens identify small RNA modulators of TRAIL-induced apoptosis pathway. *Cancer Res* 2007; 67: 10782-10788.
106. Wolf S, Mertens D, Pscherer A, Schroeter P, Winkler D, Grone HJ, Hofele C, Hemminki K, Kumar R, Steineck G, Dohner H, Stilgenbauer S and Lichter P. Ala228 variant of trail receptor 1 affecting the ligand binding site is associated with chronic lymphocytic leukemia, mantle cell lymphoma, prostate cancer, head and neck squamous cell carcinoma and bladder cancer. *Int J Cancer* 2006; 118: 1831-1835.
107. MacFarlane M, Inoue S, Kohlhaas SL, Majid A, Harper N, Kennedy DB, Dyer MJ and Cohen GM. Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. *Cell Death Differ* 2005; 12: 773-782.
108. Fernandez V, Jares P, Bea S, Salaverria I, Guino E, de Sanjose S, Colomer D, Ott G, Montserrat E and Campo E. Frequent polymorphic changes but not mutations of TRAIL receptors DR4 and DR5 in mantle cell lymphoma and other B-cell lymphoid neoplasms. *Haematologica* 2004; 89: 1322-1331.
109. Fisher MJ, Virmani AK, Wu L, Aplenc R, Harper JC, Powell SM, Rebbeck TR, Sidransky D, Gazdar AF and El Deiry WS. Nucleotide substitution in the ectodomain of trail receptor DR4 is associated with lung cancer and head and neck cancer. *Clin Cancer Res* 2001; 7: 1688-1697.
110. Dechant MJ, Fellenberg J, Scheuerpflug CG, Ewerbeck V and Debatin KM. Mutation analysis of the apoptotic "death-receptors" and the adaptors TRADD and FADD/MORT-1 in osteosarcoma tumor samples and osteosarcoma cell lines. *Int J Cancer* 2004; 109: 661-667.
111. Langsenlehner T, Langsenlehner U, Renner W, Kapp KS, Krippel P, Hofmann G, Clar H, Pummer K and Mayer R. The Glu228Ala polymorphism in the ligand binding domain of death receptor 4 is associated with increased risk for prostate cancer metastases. *Prostate* 2008; 68: 264-268.
112. Jeng YM and Hsu HC. Mutation of the DR5/TRAIL receptor 2 gene is infrequent in hepatocellular carcinoma. *Cancer Lett* 2002; 181: 205-208.
113. Lee SH, Shin MS, Kim HS, Lee HK, Park WS, Kim SY, Lee JH, Han SY, Park JY, Oh RR, Jang JJ, Han JY, Lee JY and Yoo NJ. Alterations of the DR5/TRAIL receptor 2 gene in non-small cell lung cancers. *Cancer Res* 1999; 59: 5683-5686.
114. Pai SI, Wu GS, Ozoren N, Wu L, Jen J, Sidransky D and El Deiry WS. Rare loss-of-function mutation of a death receptor gene in head and neck cancer. *Cancer Res* 1998; 58: 3513-3518.
115. Park WS, Lee JH, Shin MS, Park JY, Kim HS, Kim YS, Park CH, Lee SK, Lee SH, Lee SN, Kim H, Yoo NJ and Lee JY. Inactivating mutations of KILLER/DR5 gene in gastric cancers. *Gastroenterology* 2001; 121: 1219-1225.
116. Shin MS, Kim HS, Lee SH, Park WS, Kim SY, Park JY, Lee JH, Lee SK, Lee SN, Jung SS, Han JY, Kim H, Lee JY and Yoo NJ. Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers. *Cancer Res* 2001; 61: 4942-4946.

117. Lee SH, Shin MS, Kim HS, Lee HK, Park WS, Kim SY, Lee JH, Han SY, Park JY, Oh RR, Kang CS, Kim KM, Jang JJ, Nam SW, Lee JY and Yoo NJ. Somatic mutations of TRAIL-receptor 1 and TRAIL-receptor 2 genes in non-Hodgkin's lymphoma. *Oncogene* 2001; 20: 399-403.
118. Macartney-Coxson DP, Hood KA, Shi HJ, Ward T, Wiles A, O'Connor R, Hall DA, Lea RA, Royds JA, Stubbs RS and Rooker S. Metastatic susceptibility locus, an 8p hot-spot for tumour progression disrupted in colorectal liver metastases: 13 candidate genes examined at the DNA, mRNA and protein level. *BMC Cancer* 2008; 8: 187.
119. Addams J, Cuthbert-Heavens D, Bass S and Knowles MA. Infrequent mutation of TRAIL receptor 2 (TRAIL-R2/DR5) in transitional cell carcinoma of the bladder with 8p21 loss of heterozygosity. *Cancer Lett* 2005; 220: 137-144.
120. McDonald ER, III, Chui PC, Martelli PF, Dicker DT and El Deiry WS. Death domain mutagenesis of KILLER/DR5 reveals residues critical for apoptotic signaling. *J Biol Chem* 2001; 276: 14939-14945.
121. Bin L, Thorburn J, Thomas LR, Clark PE, Humphreys R and Thorburn A. Tumor-derived mutations in the trail receptor DR5 inhibit trail signaling through the DR4 receptor by competing for ligand binding. *J Biol Chem* 2007; 282: 28189-28194.
122. Fulda S. Caspase-8 in cancer biology and therapy. *Cancer Lett* 2009; 281: 128-133.
123. Soung YH, Lee JW, Kim SY, Nam SW, Park WS, Kim SH, Lee JY, Yoo NJ and Lee SH. Mutation of FADD gene is rare in human colon and stomach cancers. *APMIS* 2004; 112: 595-597.
124. Tur V, van der Sloot AM, Reis CR, Szegezdi E, Cool RH, Samali A, Serrano L and Quax WJ. DR4-selective Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Variants Obtained by Structure-based Design. *J Biol Chem* 2008; 283: 20560-20568.
125. van der Sloot AM, Tur V, Szegezdi E, Mullally MM, Cool RH, Samali A, Serrano L and Quax WJ. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. *Proc Natl Acad Sci U S A* 2006; 103: 8634-8639.
126. Reis CR, van der Sloot AM, Szegezdi E, Natoni A, Tur V, Cool RH, Samali A, Serrano L and Quax WJ. Enhancement of Antitumor Properties of rhTRAIL by Affinity Increase toward Its Death Receptors (dagger). *Biochemistry* 2009; 48: 2180-2191.
127. Kelley RF, Totpal K, Lindstrom SH, Mathieu M, Billeci K, DeForge L, Pai R, Hymowitz SG and Ashkenazi A. Receptor-selective mutants of apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling. *J Biol Chem* 2005; 280: 2205-2212.
128. MacFarlane M, Kohlhaas SL, Sutcliffe MJ, Dyer MJ and Cohen GM. TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. *Cancer Res* 2005; 65: 11265-11270.
129. Tolcher AW, Mita M, Meropol NJ, von MM, Patnaik A, Padavic K, Hill M, Mays T, McCoy T, Fox NL, Halpern W, Corey A and Cohen RB. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *J Clin Oncol* 2007; 25: 1390-1395.

130. Plummer R, Attard G, Pacey S, Li L, Razak A, Perrett R, Barrett M, Judson I, Kaye S, Fox NL, Halpern W, Corey A, Calvert H and de BJ. Phase 1 and pharmacokinetic study of lexatimumab in patients with advanced cancers. *Clin Cancer Res* 2007; 13: 6187-6194.
131. Pukac L, Kanakaraj P, Humphreys R, Alderson R, Bloom M, Sung C, Riccobene T, Johnson R, Fiscella M, Mahoney A, Carrell J, Boyd E, Yao XT, Zhang L, Zhong L, von KA, Shepard L, Vaughan T, Edwards B, Dobson C, Salcedo T and Albert V. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types in vitro and in vivo. *Br J Cancer* 2005; 92: 1430-1441.
132. Zeng Y, Wu XX, Fiscella M, Shimada O, Humphreys R, Albert V and Kakehi Y. Monoclonal antibody to tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) induces apoptosis in primary renal cell carcinoma cells in vitro and inhibits tumor growth in vivo. *Int J Oncol* 2006; 28: 421-430.
133. Georgakis GV, Li Y, Humphreys R, Andreeff M, O'Brien S, Younes M, Carbone A, Albert V and Younes A. Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. *Br J Haematol* 2005; 130: 501-510.
134. Marini P, Denzinger S, Schiller D, Kauder S, Welz S, Humphreys R, Daniel PT, Jendrossek V, Budach W and Belka C. Combined treatment of colorectal tumours with agonistic TRAIL receptor antibodies HGS-ETR1 and HGS-ETR2 and radiotherapy: enhanced effects in vitro and dose-dependent growth delay in vivo. *Oncogene* 2006; 25: 5145-5154.
135. Nawrocki ST, Carew JS, Douglas L, Cleveland JL, Humphreys R and Houghton JA. Histone deacetylase inhibitors enhance lexatimumab-induced apoptosis via a p21Cip1-dependent decrease in survivin levels. *Cancer Res* 2007; 67: 6987-6994.
136. Smith MR, Jin F and Joshi I. Bortezomib sensitizes non-Hodgkin's lymphoma cells to apoptosis induced by antibodies to tumor necrosis factor related apoptosis-inducing ligand (TRAIL) receptors TRAIL-R1 and TRAIL-R2. *Clin Cancer Res* 2007; 13: 5528s-5534s.
137. Adams C, Totpal K, Lawrence D, Marsters S, Pitti R, Yee S, Ross S, DeForge L, Koeppen H, Sagolla M, Compaan D, Lowman H, Hymowitz S and Ashkenazi A. Structural and functional analysis of the interaction between the agonistic monoclonal antibody Apomab and the proapoptotic receptor DR5. *Cell Death Differ* 2008; 15: 751-761.
138. Ichikawa K, Liu W, Zhao L, Wang Z, Liu D, Ohtsuka T, Zhang H, Mountz JD, Koopman WJ, Kimberly RP and Zhou T. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat Med* 2001; 7: 954-960.
139. Li J, Knee DA, Wang YZ, Zhang QX, Johnson JA, Cheng J, He H, Miller C, Li ZF, Kowal C, Eckman J, Tang B, Yuan J, Chen L, Deveraux Q, Nasoff MS and Stover D. LBY135, a novel anti-DR5 agonistic antibody induces tumor cell-specific cytotoxic activity in human colon tumor cell lines and xenografts. *Drug Dev Res* 2008; 69: 69-82.

140. Jin H, Yang R, Ross J, Fong S, Carano R, Totpal K, Lawrence D, Zheng Z, Koeppen H, Stern H, Schwall R and Ashkenazi A. Cooperation of the agonistic DR5 antibody apomab with chemotherapy to inhibit orthotopic lung tumor growth and improve survival. *Clin Cancer Res* 2008; 14: 7733-7740.
141. Estes J, Oliver P, Straughn J, Zhou T, Wang W, Grizzle W, Alvarez R, Stockard C, LoBuglio A and Buchsbaum D. Efficacy of anti-death receptor 5 (DR5) antibody (TRA-8) against primary human ovarian carcinoma using a novel ex vivo tissue slice model. *Gynecol Oncol* 2007; 105: 291-298.
142. Kendrick JE, Straughn JM, Jr., Oliver PG, Wang W, Nan L, Grizzle WE, Stockard CR, Alvarez RD and Buchsbaum DJ. Anti-tumor activity of the TRA-8 anti-DR5 antibody in combination with cisplatin in an ex vivo human cervical cancer model. *Gynecol Oncol* 2008.
143. Luster TA, Carrell JA, McCormick K, Sun D and Humphreys R. Mapatumumab and lexatumumab induce apoptosis in TRAIL-R1 and TRAIL-R2 antibody-resistant NSCLC cell lines when treated in combination with bortezomib. *Mol Cancer Ther* 2009; 8: 292-302.
144. Buchsbaum DJ, Zhou T, Grizzle WE, Oliver PG, Hammond CJ, Zhang S, Carpenter M and LoBuglio AF. Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. *Clin Cancer Res* 2003; 9: 3731-3741.
145. Straughn JM, Jr., Oliver PG, Zhou T, Wang W, Alvarez RD, Grizzle WE and Buchsbaum DJ. Anti-tumor activity of TRA-8 anti-death receptor 5 (DR5) monoclonal antibody in combination with chemotherapy and radiation therapy in a cervical cancer model. *Gynecol Oncol* 2006; 101: 46-54.
146. Greco FA, Bonomi P, Crawford J, Kelly K, Oh Y, Halpern W, Lo L, Gallant G and Klein J. Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. *Lung Cancer* 2008; 61: 82-90.
147. Hotte SJ, Hirte HW, Chen EX, Siu LL, Le LH, Corey A, Iacobucci A, MacLean M, Lo L, Fox NL and Oza AM. A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. *Clin Cancer Res* 2008; 14: 3450-3455.
148. Kanzler S, Trarbach T, Heinemann V, Köhne CH, Sneller V., Bieber FGPR and Seeber S. Results of a phase 2 trial of HGS-ETR1 (agonistic human monoclonal antibody to TRAIL receptor 1) in subjects with relapsed or refractory colorectal cancer (CRC). *Eur J Cancer* 2005; 3: (Suppl 3; abstr 630):177.
149. Leong S, Cohen RB, Gustafson DL, Langer CJ, Camidge DR, Padavic K, Gore L, Smith M, Chow LQ, von Mehren M, O'Bryant C, Hariharan S, Diab S, Fox NL, Miceli R and Eckhardt SG. Mapatumumab, an Antibody Targeting TRAIL-R1, in Combination With Paclitaxel and Carboplatin in Patients With Advanced Solid Malignancies: Results of a Phase I and Pharmacokinetic Study. *J Clin Oncol* 2009; 27: 4413-4421.
150. Mom CH, Verweij J, Oldenhuis CNAM, Gietema JA, Fox NL, Miceli R, Eskens FALM, Loos WJ, de Vries EGE and Sleijfer S. Mapatumumab, a Fully Human Agonistic Monoclonal Antibody That

- Targets TRAIL-R1, in Combination with Gemcitabine and Cisplatin: a Phase I Study. Clin Cancer Res 2009; 15: 5584-5590.
151. Younes A, Vose J, Zelenetz A, Smith M, Burris H, Ansell S, Klein J, Kumm E and Czuczman M. Results of a Phase 2 Trial of HGS-ETR1 (Agonistic Human Monoclonal Antibody to TRAIL Receptor 1) in Subjects with Relapsed/Refractory Non-Hodgkin's Lymphoma (NHL). ASH Annual Meeting Abstracts 2005; 106: 489.
 152. Camidge DR, Herbst RS, Gordon M, Eckhardt S, Kurzrock R, Durbin B, Ing J, Sager J and Mendelson D. A phase I safety and pharmacokinetic study of apomab, a human DR5 agonist antibody, in patients with advanced cancer. J Clin Oncol 2007; 25: 3582.
 153. Kindler HL, Garbo L, Stephenson J, Iezorek J, Abin T and Su M. A phase Ib study to evaluate the safety and efficacy of AMG 655 in combination with gemcitabine (G) in patients (pts) with metastatic pancreatic cancer (PC). J Clin Oncol 2009; 27.
 154. LoRusso P, Hong D, Heath E, Kurzrock R, Wang D, Hsu M, Goyal L, Wiezorek J, Storgard C and Herbst R. First-in-human study of AMG 655, a pro-apoptotic TRAIL receptor-2 agonist, in adult patients with advanced solid tumors. J Clin Oncol 2007; 25: 3538.
 155. Paz-Ares L, Sánchez Torres JM, Diaz-Padilla I, Links M, Reguart N and Boyer M. Safety and efficacy of AMG 655 in combination with paclitaxel and carboplatin (PC) in patients with advanced non-small cell lung cancer (NSCLC). J Clin Oncol 2009; 27.
 156. Rougier P, Infante J, van Laethem J, Stephenson JJ, Uronis H, Schwartzberg L and Zelenetz AD. A phase Ib/II trial of AMG 655 and panitumumab (pmab) for the treatment (tx) of metastatic colorectal cancer (mCRC): Safety results. J Clin Oncol 2009; 27.
 157. Saleh MN, Perent I, Wood TE, Posey J, Shah J, Carlisle R, Wojtowicz-Praga S and Forero-Torres A. A phase I study of CS-1008 (humanized monoclonal antibody targeting death receptor 5 or DR5), administered weekly to patients with advanced solid tumors or lymphomas. J Clin Oncol 2008; 26.
 158. Saltz L, IJ, Schwartzberg L, Stephenson J, Rocha-Lima C, Galimi F, Dillingham K, Hsu M, Wiezorek J and Fuchs C. Safety and efficacy of AMG 655 plus modified FOLFOX6 (mFOLFOX6) and bevacizumab (B) for the first-line treatment of patients (pts) with metastatic colorectal cancer (mCRC). J Clin Oncol 2009; 27.
 159. Sikic BI, Wakelee HA, von Mehren M, Lewis N, Calvert AH, Plummer ER, Fox NL, Howard T, Jones SF and Burris HA. A phase Ib study to assess the safety of lexatumumab, a human monoclonal antibody that activates TRAIL-R2, in combination with gemcitabine, pemetrexed, doxorubicin or FOLFIRI. J Clin Oncol 2007; 25.
 160. Wakelee HA, Patnaik A, Sikic BI, Mita M, Fox NL, Miceli R, Ullrich SJ, Fisher GA and Tolcher AW. Phase I and pharmacokinetic study of lexatumumab (HGS-ETR2) given every 2 weeks in patients with advanced solid tumors. Ann Oncol 2010; 21: 376-381.
 161. Chan F, Siegel R and Lenardo M. Signaling by the TNF Receptor Superfamily and T cell homeostasis. Immunity 2000; 13: 419-422.

162. Chuntharapai A, Dodge K, Grimmer K, Schroeder K, Marsters SA, Koeppen H, Ashkenazi A and Kim KJ. Isotype-dependent inhibition of tumor growth in vivo by monoclonal antibodies to death receptor 4. *J Immunol* 2001; 166: 4891-4898.
163. Muhlenbeck F, Schneider P, Bodmer JL, Schwenzer R, Hauser A, Schubert G, Scheurich P, Moosmayer D, Tschopp J and Wajant H. The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. *J Biol Chem* 2000; 275: 32208-32213.
164. Wajant H, Moosmayer D, Wuest T, Bartke T, Gerlach E, Schonherr U, Peters N, Scheurich P and Pfizenmaier K. Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. *Oncogene* 2001; 20: 4101-4106.
165. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002; 2: 420-430.
166. Natoni A, MacFarlane M, Inoue S, Walewska R, Majid A, Knee D, Stover DR, Dyer MJ and Cohen GM. TRAIL signals to apoptosis in chronic lymphocytic leukaemia cells primarily through TRAIL-R1 whereas cross-linked agonistic TRAIL-R2 antibodies facilitate signalling via TRAIL-R2. *Br J Haematol* 2007; 139: 568-577.
167. Motoki K, Mori E, Matsumoto A, Thomas M, Tomura T, Humphreys R, Albert V, Muto M, Yoshida H, Aoki M, Tamada T, Kuroki R, Yoshida H, Ishida I, Ware C and Kataoka S. Enhanced Apoptosis and Tumor Regression Induced by a Direct Agonist Antibody to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor 2. *Clin Cancer Res* 2005; 11: 3126-3135.
168. Koschny R, Walczak H and Ganten TM. The promise of TRAIL-potential and risks of a novel anticancer therapy. *J Mol Med* 2007; 85: 923-935.
169. Vitetta E and Uhr J. Monoclonal Antibodies as Agonists: An Expanded Role for Their Use in Cancer Therapy. *Cancer Res* 1994; 54: 5301-5309.
170. Higuchi H, Bronk SF, Takikawa Y, Werneburg N, Takimoto R, El Deiry W and Gores GJ. The bile acid glycochenodeoxycholate induces trail-receptor 2/DR5 expression and apoptosis. *J Biol Chem* 2001; 276: 38610-38618.
171. Poh TW, Huang S, Hirpara JL and Pervaiz S. LY303511 amplifies TRAIL-induced apoptosis in tumor cells by enhancing DR5 oligomerization, DISC assembly, and mitochondrial permeabilization. *Cell Death Differ* 2007; 14: 1813-1825.
172. Brown DA and Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 1992; 68: 533-544.
173. Rothberg KG, Ying YS, Kamen BA and Anderson RG. Cholesterol controls the clustering of the glycopospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J Cell Biol* 1990; 111: 2931-2938.
174. Simons K and Ikonen E. Functional rafts in cell membranes. *Nature* 1997; 387: 569-572.

175. Galbiati F, Razani B and Lisanti MP. Emerging themes in lipid rafts and caveolae. *Cell* 2001; 106: 403-411.
176. Mollinedo F and Gajate C. Fas/CD95 death receptor and lipid rafts: New targets for apoptosis-directed cancer therapy. *Drug Resist Updat* 2006; 9: 51-73.
177. Gajate C and Mollinedo F. Edelfosine and perifosine induce selective apoptosis in multiple myeloma by recruitment of death receptors and downstream signaling molecules into lipid rafts. *Blood* 2007; 109: 711-719.
178. Dumitru CA, Carpinteiro A, Trarbach T, Hengge UR and Gulbins E. Doxorubicin enhances TRAIL-induced cell death via ceramide-enriched membrane platforms. *Apoptosis* 2007; 12: 1533-1541.
179. Delmas D, Rebe C, Micheau O, Athias A, Gamber P, Grazide S, Laurent G, Latruffe N and Solary E. Redistribution of CD95, DR4 and DR5 in rafts accounts for the synergistic toxicity of resveratrol and death receptor ligands in colon carcinoma cells. *Oncogene* 2004; 23: 8979-8986.
180. VanOosten RL, Moore JM, Ludwig AT and Griffith TS. Depsipeptide (FR901228) enhances the cytotoxic activity of TRAIL by redistributing TRAIL receptor to membrane lipid rafts. *Mol Ther* 2005; 11: 542-552.
181. Saqr HE, Omran OM, Oblinger JL and Yates AJ. TRAIL-induced apoptosis in U-1242 MG glioma cells. *J Neuropathol Exp Neurol* 2006; 65: 152-161.
182. Psahoulia FH, Drosopoulos KG, Doubravska L, Andera L and Pintzas A. Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. *Mol Cancer Ther* 2007; 6: 2591-2599.
183. Martin S, Phillips DC, Szekely-Szucs K, Elghazi L, Desmots F and Houghton JA. Cyclooxygenase-2 inhibition sensitizes human colon carcinoma cells to TRAIL-induced apoptosis through clustering of DR5 and concentrating death-inducing signaling complex components into ceramide-enriched caveolae. *Cancer Res* 2005; 65: 11447-11458.
184. Ortiz-Ferron G, Yerbes R, Eramo A, Lopez-Perez AI, De Maria R and Lopez-Rivas A. Roscovitine sensitizes breast cancer cells to TRAIL-induced apoptosis through a pleiotropic mechanism. *Cell Res* 2008; 18: 664-676.
185. Xu L, Qu X, Zhang Y, Hu X, Yang X, Hou K, Teng Y, Zhang J, Sada K and Liu Y. Oxaliplatin enhances TRAIL-induced apoptosis in gastric cancer cells by CBL-regulated death receptor redistribution in lipid rafts. *FEBS Lett* 2009; 583: 943-948.
186. Dumitru CA and Gulbins E. TRAIL activates acid sphingomyelinase via a redox mechanism and releases ceramide to trigger apoptosis. *Oncogene* 2006; 25: 5612-5625.
187. Shaw AS. Lipid rafts: now you see them, now you don't. *Nat Immunol* 2006; 7: 1139-1142.
188. Pike LJ. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res* 2006; 47: 1597-1598.
189. Algeciras-Schimmich A, Shen L, Barnhart BC, Murmann AE, Burkhardt JK and Peter ME. Molecular ordering of the initial signaling events of CD95. *Mol Cell Biol* 2002; 22: 207-220.

190. Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J, Heinrich M, Merkel O, Ehrenschwender M, Adam D, Mentlein R, Kabelitz D and Schutze S. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. *Immunity* 2004; 21: 415-428.
191. Austin CD, Lawrence DA, Peden AA, Varfolomeev EE, Totpal K, De Maziere AM, Klumperman J, Arnott D, Pham V, Scheller RH and Ashkenazi A. Death-receptor activation halts clathrin-dependent endocytosis. *Proc Natl Acad Sci U S A* 2006; 103: 10283-10288.
192. Kohlhaas SL, Craxton A, Sun XM, Pinkoski MJ and Cohen GM. Receptor-mediated endocytosis is not required for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. *J Biol Chem* 2007; 282: 12831-12841.
193. Cheng J, Hylander BL, Baer MR, Chen X and Repasky EA. Multiple mechanisms underlie resistance of leukemia cells to Apo2 Ligand/TRAIL. *Mol Cancer Ther* 2006; 5: 1844-1853.
194. Hang HC and Bertozzi CR. The chemistry and biology of mucin-type O-linked glycosylation. *Bioorg Med Chem* 2005; 13: 5021-5034.
195. Hess D, Matsumoto A, Kim S, Marshall H and Stamler J. Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol* 2005; 6: 150-166.
196. Tang Z, Bauer JA, Morrison B and Lindner DJ. Nitrosylcobalamin promotes cell death via S nitrosylation of Apo2L/TRAIL receptor DR4. *Mol Cell Biol* 2006; 26: 5588-5594.
197. Resh M. Palmitoylation of Ligands, Receptors, and Intracellular Signaling Molecules. *Science Signaling* 2006; 2006: re14.
198. Rossin A, Derouet M, Abdel-Sater F and Hueber AO. Palmitoylation of the TRAIL receptor DR4 confers an efficient TRAIL-induced cell death signalling. *Biochem J* 2009; 419: 185-92, 2.
199. Cursi S, Rufini A, Stagni V, Condo I, Matafora V, Bachi A, Bonifazi AP, Coppola L, Superti-Furga G, Testi R and Barila D. Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *EMBO J* 2006; 25: 1895-1905.
200. De Toni E, Kuntzen C, Gerbes A, Thasler W, Sonuc N, Mucha S, Camaj P, Bruns C, Goke B and Eichhorst S. P60-c-src suppresses apoptosis through inhibition of caspase 8 activation in hepatoma cells, but not in primary hepatocytes. *J Hepatol* 2007; 46: 682-691.
201. Higuchi H, Yoon J, Grambihler A, Werneburg N, Bronk S and Gores G. Bile Acids Stimulate cFLIP Phosphorylation Enhancing TRAIL-mediated Apoptosis. *J Biol Chem* 2003; 278: 454-461.
202. Johnson T, Stone K, Nikrad M, Yeh T, Zong W, Thompson C, Nesterov A and Kraft A. The proteasome inhibitor PS-341 overcomes TRAIL resistance in Bax and caspase 9-negative or Bcl-xL overexpressing cells. *Oncogene* 2005; 22: 4953-4963.
203. Poukkula M, Kaunisto A, Hietakangas V, Denessiouk K, Katajamaki T, Johnson M, Sistonen L and Eriksson J. Rapid Turnover of c-FLIPshort Is Determined by Its Unique C-terminal Tail. *J Biol Chem* 2005; 280: 27345-27355.

204. Chen S, Liu X, Yue P, Schonthal A, Khuri F and Sun S. CCAAT/enhancer binding protein homologous protein-dependent death receptor 5 induction and ubiquitin/proteasome-mediated cellular FLICE-inhibitory protein down-regulation contribute to enhancement of Tumor Necrosis Factor-related apoptosis-inducing ligand-induced apoptosis by dimethyl-celecoxib in human non small-cell lung cancer cells. *Mol Pharm* 2007; 72: 1269-1279.
205. Kim Y, Suh N, Sporn M and Reed J. An Inducible Pathway for Degradation of FLIP Protein Sensitizes Tumor Cells to TRAIL-induced Apoptosis. *J Biol Chem* 2002; 277: 22320-22329.
206. McDonald ER, III and El Deiry WS. Suppression of caspase-8- and -10-associated RING proteins results in sensitization to death ligands and inhibition of tumor cell growth. *Proc Natl Acad Sci U S A* 2004; 101: 6170-6175.
207. Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lill JR and Ashkenazi A. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell* 2009; 137: 721-735.
208. Jesenberger V and Jentsch S. Deadly encounter: ubiquitin meets apoptosis. *Nat Rev Mol Cell Biol* 2002; 3: 112-121.
209. Lee JC and Peter ME. Regulation of apoptosis by ubiquitination. *Immunol Rev* 2003; 193: 39-47.
210. Park S, Schickel R and Peter M. Nonapoptotic functions of FADD-binding death receptors and their signaling molecules. *Curr Opin Cell Biol* 2005; 17: 610-616.
211. Boldin M, Mett I and Wallach D. A protein related to a proteasomal subunit binds to the intracellular domain of the p55 TNF receptor upstream to its death domain. *FEBS Letters* 1995; 367: 39-44.
212. Kennedy N and Budd R. Phosphorylation of FADD/MORT1 and Fas by Kinases That Associate with the Membrane-Proximal Cytoplasmic Domain of Fas. *J Immunol* 1998; 160: 4881-4888.
213. Ruiz-Ruiz C, Robledo G, Font J, Izquierdo M and Lopez-Rivas A. Protein Kinase C Inhibits CD95 (Fas/APO-1)-Mediated Apoptosis by at Least Two Different Mechanisms in Jurkat T Cells. *The J Immunol* 1999; 163: 4737-4746.
214. Sarker M, Ruiz-Ruiz C and Lopez-Rivas A. Activation of protein kinase C inhibits TRAIL-induced caspases activation, mitochondrial events and apoptosis in a human leukemic T cell line. *Cell Death Differ* 2001; 8: 172-181.
215. Meng XW, Heldebrant MP and Kaufmann SH. Phorbol 12-myristate 13-acetate inhibits death receptor-mediated apoptosis in Jurkat cells by disrupting recruitment of Fas-associated polypeptide with death domain. *J Biol Chem* 2002; 277: 3776-3783.
216. Harper N, Hughes MA, Farrow SN, Cohen GM and MacFarlane M. Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. *J Biol Chem* 2003; 278: 44338-44347.
217. Scaffidi C, Volkland J, Blomberg I, Hoffmann I, Krammer P and Peter M. Phosphorylation of FADD/ MORT1 at Serine 194 and Association with a 70-kDa Cell Cycle-Regulated Protein Kinase. *J Immunol* 2000; 164: 1236-1242.

218. Sandra F, Degli Esposti MA, Ndebele K, Gona P, Knight D, Rosenquist M and Khosravi-Far R. Tumor necrosis factor-related apoptosis-inducing ligand alters mitochondrial membrane lipids. *Cancer Res* 2005; 65: 8286-8297.
219. Lai JM, Hsieh CL and Chang ZF. Caspase activation during phorbol ester-induced apoptosis requires ROCK-dependent myosin-mediated contraction. *J Cell Sci* 2003; 116: 3491-3501.
220. Frankel SK, Van Linden AA and Riches DW. Heterogeneity in the phosphorylation of human death receptors by p42(mapk/erk2). *Biochem Biophys Res Commun* 2001; 288: 313-320.
221. Soderstrom TS, Poukkula M, Holmstrom TH, Heiskanen KM and Eriksson JE. Mitogen-activated protein kinase/extracellular signal-regulated kinase signaling in activated T cells abrogates TRAIL-induced apoptosis upstream of the mitochondrial amplification loop and caspase-8. *J Immunol* 2002; 169: 2851-2860.
222. Tran SE, Holmstrom TH, Ahonen M, Kahari VM and Eriksson JE. MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. *J Biol Chem* 2001; 276: 16484-16490.
223. Izeradjene K, Douglas L, Delaney A and Houghton JA. Influence of casein kinase II in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human rhabdomyosarcoma cells. *Clin Cancer Res* 2004; 10: 6650-6660.
224. Izeradjene K, Douglas L, Delaney AB and Houghton JA. Casein kinase I attenuates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by regulating the recruitment of fas-associated death domain and procaspase-8 to the death-inducing signaling complex. *Cancer Res* 2004; 64: 8036-8044.
225. Izeradjene K, Douglas L, Delaney A and Houghton JA. Casein kinase II (CK2) enhances death-inducing signaling complex (DISC) activity in TRAIL-induced apoptosis in human colon carcinoma cell lines. *Oncogene* 2005; 24: 2050-2058.
226. Cowling V and Downward J. Caspase-6 is the direct activator of caspase-8 in the cytochrome c-induced apoptosis pathway: absolute requirement for removal of caspase-6 prodomain. *Cell Death Differ* 2002; 9: 1046-1056.
227. Hao C, Beguinot F, Condorelli G, Trencia A, Van Meir EG, Yong VW, Parney IF, Roa WH and Petruk KC. Induction and intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis in human malignant glioma cells. *Cancer Res* 2001; 61: 1162-1170.
228. Araujo H, Danziger N, Cordier J, Glowinski J and Chneiweiss H. Characterization of PEA-15, a major substrate for protein kinase C in astrocytes. *J Biol Chem* 1993; 268: 5911-5920.
229. Trencia A, Perfetti A, Cassese A, Vigliotta G, Miele C, Oriente F, Santopietro S, Giacco F, Condorelli G, Formisano P and Beguinot F. Protein kinase B/Akt binds and phosphorylates PED/PEA-15, stabilizing its antiapoptotic action. *Mol Cell Biol* 2003; 23: 4511-4521.
230. Xiao C, Yang BF, Asadi N, Beguinot F and Hao C. Tumor necrosis factor-related apoptosis-inducing ligand-induced death-inducing signaling complex and its modulation by c-FLIP and PED/PEA-15 in glioma cells. *J Biol Chem* 2002; 277: 25020-25025.

231. Estelles A, Yokoyama M, Nothias F, Vincent JD, Glowinski J, Vernier P and Chneiweiss H. The major astrocytic phosphoprotein PEA-15 is encoded by two mRNAs conserved on their full length in mouse and human. *J Biol Chem* 1996; 271: 14800-14806.
232. Kubes M, Cordier J, Glowinski J, Girault JA and Chneiweiss H. Endothelin induces a calcium-dependent phosphorylation of PEA-15 in intact astrocytes: identification of Ser104 and Ser116 phosphorylated, respectively, by protein kinase C and calcium/calmodulin kinase II in vitro. *J Neurochem* 1998; 71: 1307-1314.
233. Li Y, Wang H, Wang Z, Makhija S, Buchsbaum D, LoBuglio A, Kimberly R and Zhou T. Inducible Resistance of Tumor Cells to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor 2-Mediated Apoptosis by Generation of a Blockade at the Death Domain Function. *Cancer Res* 2006; 66: 8520-8528.
234. Sun M, Song L, Li Y, Zhou T and Jope RS. Identification of an antiapoptotic protein complex at death receptors. *Cell Death Differ* 2008; 15: 1887-1900.
235. LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S and Korneluk RG. IAP-targeted therapies for cancer. *Oncogene* 2008; 27: 6252-6275.
236. Al Zoubi A, Efimova E, Kaithamana S, Martinez O, El-Idrissi ME-A, Dogan R and Prabhakar B. Contrasting effects of IG20 and its splice isoforms, MADD and DENN-SV, on Tumor Necrosis Factor alpha -induced apoptosis and activation of caspase-8 and -3. *J Biol Chem* 2001; 276: 47202-47211.
237. Ramaswamy M, Efimova EV, Martinez O, Mulherkar NU, Singh SP and Prabhakar BS. IG20 (MADD splice variant-5), a proapoptotic protein, interacts with DR4/DR5 and enhances TRAIL-induced apoptosis by increasing recruitment of FADD and caspase-8 to the DISC. *Oncogene* 2004; 23: 6083-6094.
238. Schievella A, Chen J, Graham J and Lin L. MADD, a Novel Death Domain Protein That Interacts with the Type 1α Tumor Necrosis Factor Receptor and Activates Mitogen-activated Protein Kinase. *J Biol Chem* 1997; 272: 12069-12075.
239. Efimova EV, Al Zoubi AM, Martinez O, Kaithamana S, Lu S, Arima T and Prabhakar BS. IG20, in contrast to DENN-SV, (MADD splice variants) suppresses tumor cell survival, and enhances their susceptibility to apoptosis and cancer drugs. *Oncogene* 2004; 23: 1076-1087.
240. Mulherkar N, Ramaswamy M, Mordi DC and Prabhakar BS. MADD//DENN splice variant of the IG20 gene is necessary and sufficient for cancer cell survival. *Oncogene* 2006; 25: 6252-6261.
241. Mulherkar N, Prasad K and Prabhakar B. MADD/DENN Splice Variant of the IG20 Gene Is a Negative Regulator of Caspase-8 Activation: Knockdown enhances TRAIL-induced apoptosis of cancer cells. *J Biol Chem* 2007; 282: 11715-11721.
242. Mori S, Murakami-Mori K, Nakamura S, Ashkenazi A and Bonavida B. Sensitization of AIDS-Kaposi's Sarcoma Cells to Apo-2 Ligand-Induced Apoptosis by Actinomycin D. *J Immunol* 1999; 162: 5616-5623.

243. Murakami-Mori K, Mori S, Bonavida B and Nakamura S. Implication of TNF Receptor-I-Mediated Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2) Activation in Growth of AIDS-Associated Kaposi's Sarcoma Cells: A Possible Role of a Novel Death Domain Protein MADD in TNF- α -Induced ERK1/2 Activation in Kaposi's Sarcoma Cells. *J Immunol* 1999; 162: 3672-3679.
244. Ren YG, Wagner KW, Knee DA, Aza-Blanc P, Nasoff M and Deveraux QL. Differential regulation of the TRAIL death receptors DR4 and DR5 by the signal recognition particle. *Mol Biol Cell* 2004; 15: 5064-5074.
245. Keenan RJ, Freymann DM, Stroud RM and Walter P. The signal recognition particle. *Annu Rev Biochem* 2001; 70: 755-775.
246. Pool MR, Stumm J, Fulga TA, Sinning I and Dobberstein B. Distinct modes of signal recognition particle interaction with the ribosome. *Science* 2002; 297: 1345-1348.

Downregulation of active caspase 8 as a mechanism of acquired TRAIL-resistance in mismatch repair-proficient colon carcinoma cell lines

Caroline M.M. van Geelen¹

Bodvaël Pennarun^{1,2}

Wytske Boerma-van Ek²

Phuong T.K. Le¹

Diana C.J. Spierings¹

Elisabeth G.E. de Vries¹

Steven de Jong¹

Departments of ¹Medical Oncology, ²Gastroenterology,
University Medical Center Groningen,
University of Groningen, the Netherlands

Submitted for publication

Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers the apoptotic cascade in various colon cancer cell lines after binding to the membrane receptors DR4 and DR5. However, not all cancer cell lines are sensitive to the therapeutic recombinant human TRAIL (rhTRAIL). To investigate the causes of TRAIL resistance in colon cancer cell lines, models have been developed, mostly in mismatch repair-deficient cells. These cells are prone to mutations in genes containing tandem repeat, including pro-apoptotic protein Bax. We therefore investigated the mechanism underlying TRAIL resistance acquisition in a mismatch repair-proficient colon carcinoma cell line. The TRAIL-resistant cell line SW948-TR was established from the TRAIL-sensitive cell line SW948 by continuous exposure to rhTRAIL, and exhibited 140 fold less sensitivity to rhTRAIL in a cell viability assay. Resistance was stable for over a year in the absence of rhTRAIL. Both cell lines had similar TRAIL receptor cell membrane expression levels. Treatment with the protein synthesis inhibitor cycloheximide sensitized SW948-TR to rhTRAIL-induced apoptosis, indicating that the functionality of the TRAIL receptors was maintained. In SW948-TR, procaspase 8 protein levels but not mRNA levels were notably lower than in SW948. Downregulation of c-FLIP with short interfering RNA (siRNA) sensitized SW948-TR cells to rhTRAIL while caspase 8 siRNA decreased rhTRAIL sensitivity in SW948, indicating the importance of the caspase 8/c-FLIP ratio. Proteasome inhibition with MG132 did not restore basic procaspase 8 levels but stabilized cleaved caspase 8 in rhTRAIL-treated SW948-TR cells. Altogether, our results suggest that colon cancer cells can acquire rhTRAIL resistance by primarily reducing the basal procaspase 8/c-FLIP ratio and by increasing active caspase 8 degradation after rhTRAIL treatment. Proteasome inhibitors can effectively overcome acquired rhTRAIL resistance in mismatch repair-proficient colon cancer cells.

Introduction

TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) is a member of the TNF family with interesting anti-cancer activity (1,2). Binding of TRAIL to its two apoptosis-inducing receptors DR4 and DR5 induces formation of a complex called the death-inducing signaling complex (DISC), leading to caspase-dependent stimulation of the extrinsic pathway of apoptosis. The therapeutic agent recombinant human (rh)TRAIL has been shown to induce apoptosis in many types of human cancer cell lines *in vitro*, and also prevents tumor growth in preclinical models *in vivo*. By activating both the extrinsic and the intrinsic pathway of apoptosis, combination treatment of rhTRAIL and chemotherapy can also induce apoptosis in drug-resistant cell lines both *in vitro* and *in vivo* in human tumor xenograft mouse models. A phase I study has consequently been initiated with rhTRAIL and demonstrated safety of this agent (3-5). In a phase II study in heavily pre-treated colon cancer patients, the best response to an anti-DR4 antibody was stable disease in 32% cases (6).

About 60% of cancer cell lines are moderately to completely resistant to rhTRAIL *in vitro* (7). Understanding the molecular basis of cancer cell resistance to TRAIL-induced apoptosis may hold the key to optimal TRAIL receptor-targeted combination therapies and the development of novel treatment initiatives. Several key components for TRAIL sensitivity have been identified. This includes downstream pro- and anti-apoptotic proteins of the Bcl-2 family such as Bax, Bak and Bcl-2, proteins inhibiting efficient DISC formation such as c-FLIP and finally caspase inhibitors such as X-linked inhibitor of apoptosis protein (XIAP)(8). In DNA mismatch repair (MMR)-deficient tumor colon cancer cell line, the acquisition of TRAIL resistance after extended exposure to the ligand was linked to the accumulation of Bax mutations resulting in Bax^{-/-} phenotype, both *in vitro* and *in vivo* (9). MMR deficiency leads to micro-satellite instability (MSI). MSI-positive tumors have a high rate of frame-shift mutations in a number of genes containing tandem repeat sequences, including the pro-apoptotic gene Bax (10,11). Apart from hereditary non-polyposis colon cancer tumors, which accounts for approximately 2-5% of colorectal cancer cases, MSI as a result of MMR deficiency is present in around 15% of sporadic colon tumors (12). Because the majority of the colon cancers are MMR-proficient, this group of tumors deserves attention as well. By retroviral transfection of a human cDNA library in the TRAIL-sensitive colon cancer

cells line SW480, others previously reported that FLIP_s and Bcl-X_L overexpression could induce TRAIL resistance (13). To further identify the components for rhTRAIL sensitivity in MMR-proficient colon carcinoma cells, we established a rhTRAIL resistant sub-line by prolonged exposure of the previously characterized TRAIL-sensitive SW948 colon carcinoma cell line (14,15). The mechanisms of TRAIL resistance were analyzed in this distinct isogenic background.

Materials and Methods

Reagents

RPMI 1640 medium was obtained from Life Technologies (Breda, the Netherlands) and fetal calf serum (FCS) from Bodinco BV (Alkmaar, the Netherlands). 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT)-solution, cycloheximide and actinomycin D were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). RhTRAIL was produced non-commercially in cooperation with IQ-Corporation (Groningen, the Netherlands) following a protocol described earlier (7). The TRAIL-receptor antibodies used for flow cytometry were obtained from Immunex (Seattle, WA). Caspase 9 inhibitor zLEHD-fmk, caspase 8 inhibitor zIETD-fmk, broad-spectrum caspase inhibitor zVAD-fmk and proteasome inhibitor MG132 were obtained from Calbiochem (Breda, the Netherlands). Oligofectamine reagent was purchased from Invitrogen BV (Breda, the Netherlands). The NFκB inhibitor SN-50 was obtained from Biomol (Tebu-bio, Heerhugowaard, the Netherlands).

Cell lines

The TRAIL-sensitive colon carcinoma SW948 cell line was cultured in Leibovitz L15-RPMI 1640 (1:1) enriched with 10% FCS, 0.05 M pyruvate, 0.1 M glutamine and 0.025% β-mercaptoethanol at 37°C in a humidified atmosphere with 5% CO₂. SW948 was harvested by treatment with protease XXIV for 5-10 min at 37 °C. In order to obtain a TRAIL-resistant sub-line, SW948 was exposed continuously to 1.0 µg/ml rhTRAIL and incubated at 37°C to allow resistant cells to grow. After 14 days these cells were cultured in the presence of 2.5 µg/ml rhTRAIL twice a week. After three months the stable TRAIL-resistant cell line SW948-TR was established and cultured similarly to its parental cell line. In the absence of rhTRAIL in the culture media SW948-TR remains

resistant to rhTRAIL for at least 75 passages. Based on cell viability assay, this cell line is at least 140 fold more resistant to rhTRAIL than the sensitive parental cell line.

Sub-cloning of SW948-TR

SW948-TR was harvested by treatment with protease XXIV for 5-10 min at 37°C, washed once in Leibovitz L15-RPMI 1640 (1:1) without FCS. A single cell suspension was obtained by resuspending the cells repeatedly through an 18-gauge needle.

Cells were diluted in two volumes HAM-DME (1:1) with 20% FCS and one volume conditioned Leibovitz L15-RPMI 1640 (1:1) medium with 10% FCS. The cell suspension was kept at 37°C, then 1/10 volume of pre-warmed agarose (final concentration 0.3%) was added and carefully mixed. Two ml cell suspension was added to each Petri dish (200 or 20 cells) which was kept at 4°C for 1 h. Then, Petri dishes were placed at 37°C in a humidified atmosphere with 5% CO₂. After 14 days individual visible clones were transferred to a 24-wells plate and cultured in Leibovitz L15-RPMI 1640 (1:1) medium with 10% FCS. Growing clones were harvested by treatment with protease XXIV for 5-10 min at 37 °C and cultured as described for SW948-TR.

An alternative sub-cloning approach was minimal dilution of SW948-TR in two volumes Leibovitz L15-RPMI 1640 (1:1) medium with 10% FCS and one volume conditioned Leibovitz L15-RPMI 1640 (1:1) medium with 10% FCS. Cell suspension was diluted and thereafter 100 µl was added to each well of a 96-well plate. After 14 days, cells from wells with single colony were harvested with protease XXIV for 5-10 min at 37°C, transferred to a well of a 24-well plate, and incubated as described above.

Cytotoxicity assay

The microculture tetrazolium assay was used to determine cytotoxicity. SW948 and SW948-TR cells were incubated in a total volume of 200 µl. Treatment consisted of continuous incubation with various rhTRAIL concentrations. After an incubation period of 96 h, 20 µl of MTT-solution (5 mg/ml phosphate buffered saline (PBS): 6.4 mM Na₂HPO₄; 1.5 mM KH₂PO₄; 0.14 mM NaCl; 2.7 mM KCl; pH=7.2)) was added for 3.75 h. Subsequently, plates were centrifuged and the supernatant aspirated. After dissolving the formazan crystals by adding dimethyl sulfoxide (Merck, Amsterdam, the Netherlands), plates were read immediately at 520 nm using a microtiter well spectrometer (Bio-Rad microplate reader, Bio-Rad laboratories BV, Veenendaal, the

Netherlands). Controls consisted of media without cells. Cell survival was defined as the growth of treated cells compared to untreated cells. The IC₅₀ was defined as the drug concentration inhibiting survival by 50%. Mean cytotoxicity was calculated from three independent experiments each performed in quadruplicate.

SDS-polyacrylamide gel electrophoresis and Western blotting

Preparation of protein lysates and Western blot analysis was performed as described previously (15). To detect poly-ADP-ribose-polymerase (PARP), FADD, X-linked inhibitor of apoptosis protein (XIAP), FLICE-like inhibitory protein (FLIP), caspase 3, caspase 9, caspase 8, caspase 10, DR4, DR5, Bid, Bax, Bak, Bcl-X_L, Bcl-2, Bid and actin the following antibodies were used: rabbit anti-PARP from Roche applied science (Mannheim, Germany), rabbit mouse-anti-FADD and mouse-anti-XIAP from Transduction Laboratories (Lexington, KY), rabbit-anti-caspase 3, rabbit-anti-Bid and rabbit-anti-caspase 9 from Pharmingen (Becton Dickinson, Erebodegem-Aalst, Belgium), goat-anti-DR4, mouse-anti-Bcl-2, rabbit-anti-BclX_L, rabbit-anti-Bax (N20), goat-anti-Bak (N20) from Santa Cruz Biotechnology (Santa Cruz, CA) rabbit-anti-DR5 and rabbit-anti-survivin from Oncogene Research Products (Calbiochem-Novabiochem, Germany). Mouse anti-caspase 8 was purchased from Cell Signaling Technology (Leusden, the Netherlands). Mouse-anti-caspase 10, rabbit-anti-cIAP-1 and rabbit-anti-cIAP-2 were obtained from R&D systems (Abingdon, UK). Mouse-anti-FLIP NF6 was kindly provided by Dr. M. Peter (Chicago, IL). Mouse-anti-actin was obtained from ICN Biomedicals (Zoetermeer, the Netherlands). The secondary antibodies were labeled with horseradish peroxidase (all from DAKO, Glostrup, Denmark) and chemiluminescence was detected using the ECL-chemiluminescence kit or with the Lumi-Light Plus Western blotting kit (Roche Diagnostics, Mannheim, Germany). Western blot analyses were performed at least three times. Protein concentrations were determined with the Bradford assay. In all experiments samples containing 15 or 20 µg lysate were used, and membranes were stained with Ponceau S to check for equal protein loading.

Flow cytometry

Analysis of TRAIL-receptor membrane expression was performed with a flow cytometer (Epics Elite, Coulter-Electronics, Hialeah, FL) and cells were stained as described earlier (15). The following antibodies were used: for DR4, huTRAILR1-M271, for DR5,

huTRAILR2-M413, for DcR1, huTRAILR3-M430, and for DcR2, huTRAILR4-M444. Membrane receptor expression is shown as mean fluorescent intensity (MFI) of all analyzed cells. Membrane expression was observed as an increase in fluorescence intensity for the whole analyzed cell population. All experiments were performed at least three times.

Apoptosis assay

For each cell line 10,000 cells were seeded in 96-well plates. Apoptosis was identified by staining nuclear chromatin with acridine orange (AO), identifying morphological changes by fluorescence microscopy. Apoptosis was expressed as percentage apoptotic cells in a culture. Apoptosis experiments were performed at least three times.

RNA interference

Small interfering RNAs (siRNAs) specific for human c-FLIP were designed conforming to the sequence AA(N19)TT, where AA and TT are present in the c-FLIP open reading frame at a spacing of 19 nucleotides. Double-stranded RNA molecules specific for c-FLIP (sense: 5'-GAGGUAAGCUGUCUGUCGGdTdT-3', anti-sense: 5'-CCGACAGACAGCUUACCUCdTdT-3') or caspase 8 (sense: 5'-CUACCAGAAAGGUAUACCUdTdT-3', anti-sense: 5'-AGGUAUACCUUUCUGGUAGdTdT-3') were synthesized by Eurogentec (Seraing, Belgium). Single stranded RNA molecules specific for the luciferase (Luc) gene were used as control (16). The sequences for Luc RNA molecules were 5'-CUUACGCUGAGUACUUCGAdTdT-3' (sense) and 5'-UCGAAGUACUACGCUAA GdTdT-3' (antisense). To form RNA duplexes, 20 μ M of both single-stranded RNAs were incubated in annealing buffer supplied by Eurogentec (50 mM Tris, pH 7.5-8.0, 100 mM NaCl in RNase-free distilled water) for 1-5 min at 90°C and cooled down to room temperature in approximately 1 h. Cells were harvested with protease and transfected in 6 wells plates (0.3×10^6 cells/well) with 2.5-10 μ l of 20 μ M siRNA duplexes using oligofectamine reagent according to the manufacturer's instructions. The next day, cells were harvested and seeded in 96- or 6-well plates for apoptosis assay or protein isolation respectively. After 48 h siRNA transfected cells were incubated with 0.1 μ g/ml rhTRAIL for 5 h. After treatment, the percentage apoptosis was determined by AO apoptosis assay or the cells were lysed for protein analysis.

Real Time PCR

Total RNA was isolated by guanidine isothiocyanate-phenol-chloroform extraction using TRIzol (Invitrogen, Breda, the Netherlands) according to the manufacturer's protocol. Total RNA was purified with the RNeasy mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturer's instructions. Trace amounts of DNA contamination were removed by on-column DNase I digestion following the manufacturer's recommendations.

Yield and quality of the total purified RNA was assessed by measuring A260/280 nm and 260/230 nm ratio on a nanodrop ND-1000 spectrophotometer (Nanodrop, Isogen Life Science B.V., IJsselstein, the Netherlands) and by RNA gel electrophoresis visualization of 18S and 28S rRNA bands. cDNA was synthesized from 800 ng purified RNA as described by the manufacturer's protocol (Life Technologies, Breda, the Netherlands) using oligo (dT)₁₁ primers and MMLV transcriptase. Prior to the RT-PCR, purity and integrity of the synthesized cDNA was examined by qualitative RT-PCR for the housekeeping reference gene coding for the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real time RT-PCR was performed in 96-well plates using the SYBR Green method on a MyiQ real time detection system (all from BioRad) using GAPDH as an internal control. A gradient RT-PCR was performed to assess primer specificity and to optimize annealing temperature (T_{ann}) for each set of gene specific primers. The sequences for the primers used in real time PCR of caspase 8 were GGAGCTGCTCTTCCGAATTA (forward) and GCAGGTTCATGTCATCATCC (reverse), for FLIP and, and those of GAPDH were CACCACCATGGAGAAGGCTGG (forward) and CCAAAGTTGTCATGGATGACC (reverse). Amplification of the samples was carried out in triplicate in a final reaction volume of 25 μ l, containing 12.5 μ l IQ SYBR Green Supermix (BioRad), 1 μ l of each gene specific primer (5 μ M) and 5 μ l cDNA (1:50). The thermocycling program used for each real time RT-PCR consisted of an initial 3 min denaturation at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C, 20 s primer annealing at the primer specific T_{ann} and 30 s fragment elongation at 72 °C. Fluorescence data was acquired during the fragment elongation step at 72 °C. Presence of unique reaction products was determined from the melting curves obtained at the end of 40 cycles of amplification.

To determine RT-PCR efficiency and initial starting quantity of the samples, a standard curve was generated using a 1:3 serial dilution from total starting cDNA

sample. Water controls were included to check for DNA contamination. Differences in the amount of starting cDNA between samples were corrected using GAPDH as a housekeeping reference gene.

Statistics

Statistic analysis was performed using the Student's t-test. P values ≤ 0.05 were considered to be significant.

Results

Characteristics of the rhTRAIL-resistant cell line

The TRAIL-resistant sub-line (SW948-TR) was established from the parental colon carcinoma cell line SW948 following incubation with increasing concentrations of rhTRAIL for three months. The differences in rhTRAIL sensitivity within this isogenic cell model are shown in Figure 1A. SW948 is an extremely sensitive to rhTRAIL with an IC₅₀ of 0.007 $\mu\text{g/ml}$ whereas SW948-TR is resistant to high concentrations of rhTRAIL, or 140 fold less sensitive than its parental cell line. SW948 cells were also more sensitive to rhTRAIL-induced apoptosis than SW948-TR cells as determined in an apoptosis assay (Figure 1B). The $\sim 30\%$ surviving fraction is consistent with previous model of TRAIL resistance in cancer cell lines treated with TRAIL for several months (17,18).

TRAIL receptor membrane expression was evaluated in both cell lines to examine whether TRAIL receptors played a role in the acquisition of TRAIL resistance (Figure 1C). The cell lines showed similar DR4 ($p = 0.998$) and DR5 ($p = 0.109$) membrane expression. DcR1 and DcR2 were not or hardly expressed on the cell surface of both cell lines. Western blotting was performed to determine whether the acquisition of TRAIL resistance in SW948-TR cells was caused by a change in basal protein expression levels of DISC components, Bcl-2 family members or members of the inhibitor of apoptosis protein (IAP) family (Figure 1D). Total DR4 and DR5 levels were similar between the two cell lines. No differences in FADD, Bid, Bax, Bak, Bcl-2, Bcl-X_L, or caspase 9 were observed between SW948 and SW948-TR. Members of the IAP family, namely survivin, c-IAP1 and c-IAP2 were similarly expressed in both cell lines. Procaspase 8 levels were, however, lower and procaspase 10 levels slightly higher in SW948-TR than in SW948.

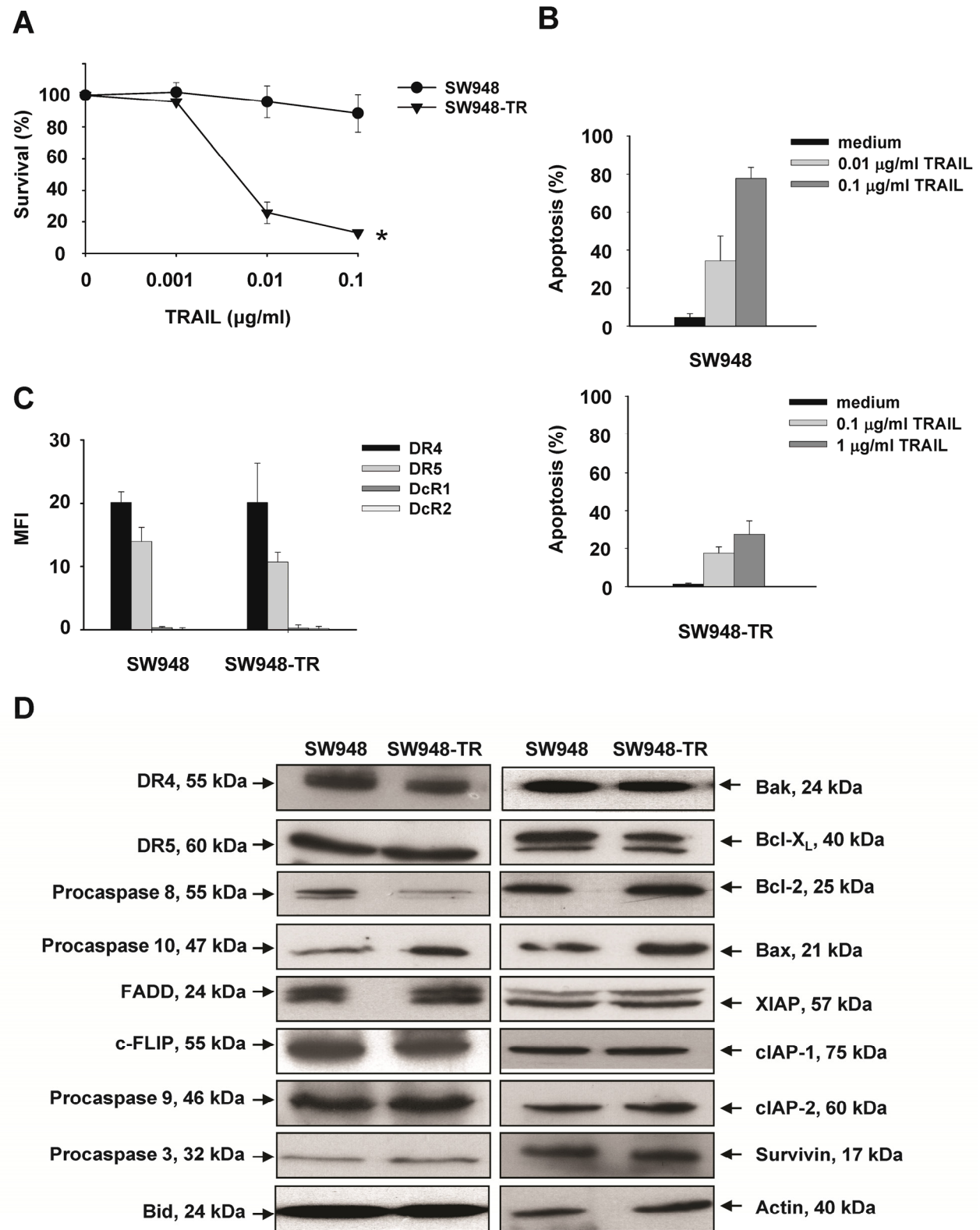


Figure 1: The SW948-TR cell line is less sensitive to rhTRAIL than its parental SW948 cell line and expressed lower procaspase 8 protein levels. **A:** Survival (%) of SW948 (closed square) and SW948-TR (closed triangle), after continuous incubation with rhTRAIL as measured by cytotoxicity assays. Values are mean \pm SD of at least three independent experiments. *: SW948 differs from SW948-TR at 0.01-1 μ g/ml rhTRAIL ($p \leq 0.05$). **B:** Apoptosis (%) of SW948 (top) and SW948-TR (bottom), after incubation with various concentrations of rhTRAIL for 5 h, as assessed following AO staining. Values are mean \pm SD of at least three independent experiments. **C:** Basic membrane expression of the TRAIL receptors DR4 (black bars), DR5 (dark gray bars), DcR1 (white bars), and DcR2 (light gray bars) in SW948 and SW948-TR. Receptor expression was detected as mean fluorescence intensity (MFI). Values are mean \pm SD of at least three independent experiments. **D:** Western blot analysis showing basic expression levels of several proteins involved in apoptosis for SW948 and SW948-TR. One representative of at least three different experiments is shown. Actin is shown as loading control.

The protein synthesis inhibitor cycloheximide sensitizes SW948-TR cells to rhTRAIL in a caspase 8-dependent manner

In SW948-TR, incubation with rhTRAIL induced apoptosis in approximately 30% of cells. The protein synthesis inhibitor cycloheximide strongly sensitized SW948-TR cells to rhTRAIL (Figure 2A). The levels of apoptosis reached in presence of cycloheximide were similar to the levels of apoptosis initially found in the parental cell line (compare with Figure 1B, top panel). These results demonstrate that the TRAIL receptor-mediated apoptotic pathway is still functional in SW948-TR cells.

Caspase 8 activation is a crucial step in death receptor-mediated apoptosis. To study whether inhibition of TRAIL-induced apoptosis in SW948-TR originates upstream, downstream, or at the level of caspase 8, the cleavage of caspase 8 and Bid in response to rhTRAIL was studied by Western blotting. Caspase 8 activation was represented by either the intermediate caspase 8 product (p45/47) or the active caspase 8 (p18) sub-units, and activation of Bid by disappearance of the full length protein. Exposure of SW948 to rhTRAIL for 5 h induced cleavage of procaspase 8 as well as Bid activation (Figure 2B, lane 2). In the presence of cycloheximide and rhTRAIL, caspase 8 cleavage and Bid activation were also observed in SW948-TR (Figure 2B, lane 5). Since the TRAIL-sensitive fraction of approximately 30% in SW948-TR may have complicated our observations (see also Figure 2A), the apoptotic (A) and the surviving (S) fraction of SW948-TR were separated after exposure to rhTRAIL for 5 h. High levels of cleaved caspase 8 and complete Bid activation were detected in the apoptotic fraction (Figure 2B,

lane 7). In contrast, Bid was not cleaved in the surviving fraction where pro- and active caspase 8 were almost undetectable (lane 6 in Figure 2B). These results strongly suggest that resistance is initiated at the level of caspase 8 in SW948-TR, through downregulation of pro- and/or active caspase 8 levels.

In presence of cycloheximide, both the caspase 8-specific inhibitor zIETD-fmk and the pan-caspase inhibitor zVAD-fmk inhibited TRAIL-induced apoptosis in the resistant cell line (Figure 2C), indicating the importance of caspases in TRAIL-induced apoptosis following cycloheximide treatment. In contrast, the caspase 9 inhibitor zLEHD-fmk could not inhibit TRAIL-induced apoptosis in SW948-TR (Figure 2D). Thus, in presence of cycloheximide, enough active caspases can be generated without the need for amplification via caspase 9 downstream of the mitochondria (19).

Similar phenotypes in sub-clones of SW948-TR

Since cell cycle distribution in the surviving fraction was similar to that of untreated cells, we excluded the possibility that the apoptotic fraction consisted of cells in a specific phase of the cell cycle (data not shown). To test whether SW948-TR consisted of a heterogeneous cell population causing differential expression of caspase 8 in 30% of the cells, 15 sub-clones were isolated using minimal dilution and soft agar sub-cloning methods. Effects of rhTRAIL incubation on cell survival in SW948, SW948-TR and four randomly-chosen clones are shown in Figure 3A. Screening for DISC protein expression, we found no difference in membrane TRAIL receptors, pro-caspase 8 and c-FLIP protein expression levels between the sub-lines (Figure 3 B-C). Remarkably, most sub-clones showed apoptosis in a range of 20-40% after incubation with rhTRAIL alone, and were completely sensitized to rhTRAIL by cycloheximide (CHX, Figure 3D). Clone 4 exhibited slightly higher rhTRAIL sensitivity in survival and apoptosis assay (Figure 3A and D). This may be explained by the fact that clone 4 exhibited higher caspase 8 expression and similar c-FLIP expression compared to other clones (for example, compare clone 1 and 4 in Figure 3B). The fact that we could not isolate individual stable TRAIL-sensitive and TRAIL-resistant clones suggest that the ~ 30% apoptosis seen in SW948-TR cells after rhTRAIL treatment is a general property of the cell population. The existence of an apoptotic fraction might partly be explained by small cell-to-cell variations in caspase 8 and FLIP levels in favor of apoptosis.

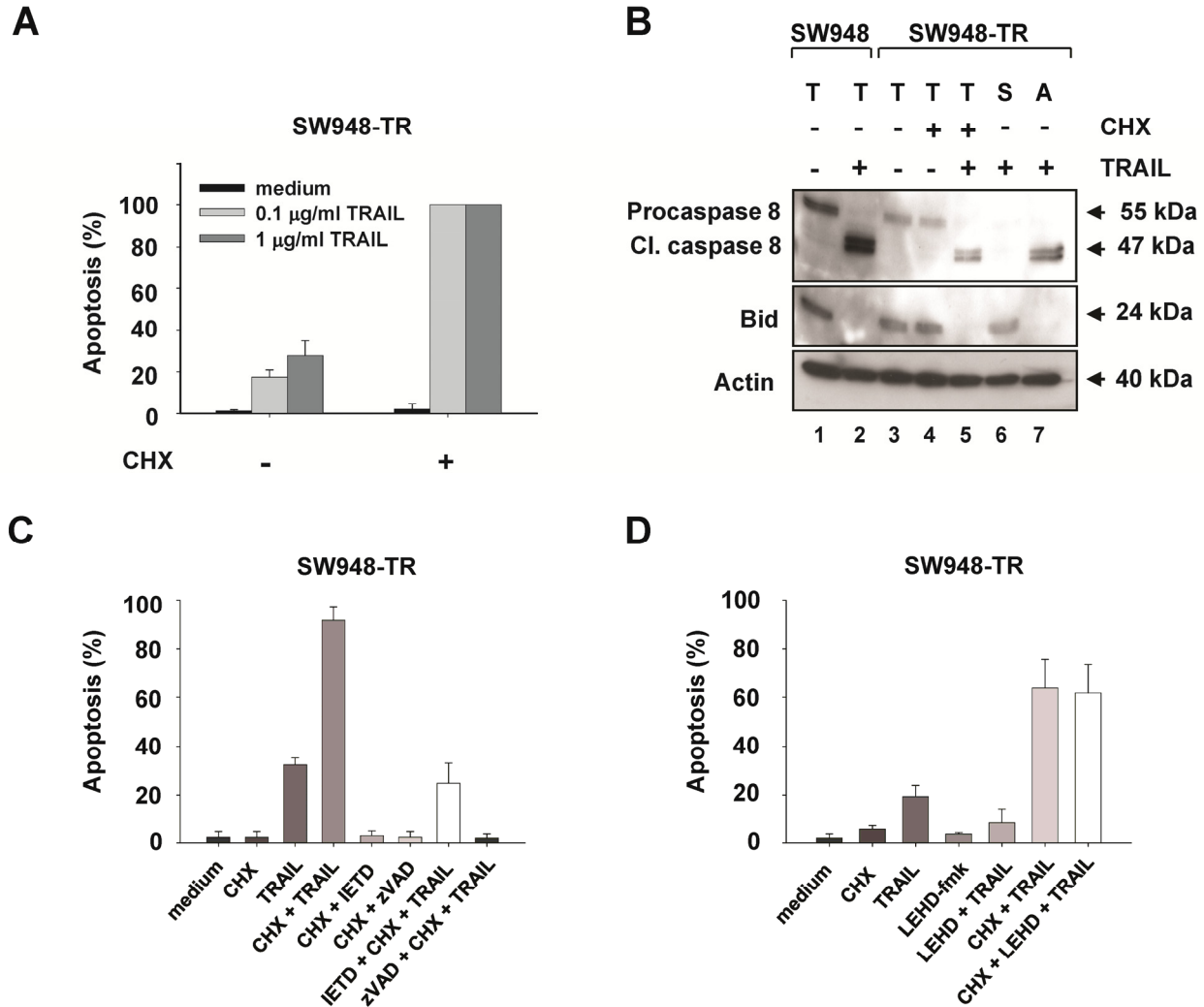


Figure 2: The protein synthesis inhibitor cycloheximide (CHX) sensitizes SW948-TR cells to rhTRAIL-induced apoptosis in a caspase 8-dependent fashion. **A:** Sensitization of SW948-TR cells to rhTRAIL-induced apoptosis by CHX, as determined by AO staining. Cells were exposed to 0.1 or 1.0 µg/ml rhTRAIL for 24 h with or without pre-incubation with 5 µg/ml CHX for 1 h. Values are mean ± SD of at least three independent experiments. **B:** Western blot analysis of caspase 8 activation and Bid cleavage in SW948 and SW948-TR cells. Cells were treated with 5 µg/ml CHX, 0.1 µg/ml rhTRAIL alone or in combination. Total cell lysates were loaded (T) and for the SW948-TR the surviving sub-fraction (S) was separated from the apoptotic (A) cell fraction. Procaspase 8 (55 kD) is cleaved in an intermediate product (cleaved caspase 8, Cl. caspase 8) (47 kD) and an active p18 product (18 kD). Bid (24 kD) is cleaved in tBid (not detectable) by active caspase 8. Bands of interest are indicated with arrows. **C:** Caspase 8-dependent apoptosis in CHX-treated SW948-TR cells. Cells were pre-incubated for 1 h with 5 µg/ml cycloheximide and/or 50 µM zIETD-fmk or 50 µM zVAD-fmk before treatment with 0.1 µg/ml rhTRAIL for 4 h. **D:** SW948-TR cells were pre-incubated for 1 h with 5 µg/ml CHX and/or 50 µM zLEHD-fmk before 4 h-treatment with 0.1 µg/ml rhTRAIL and AO staining for apoptosis.

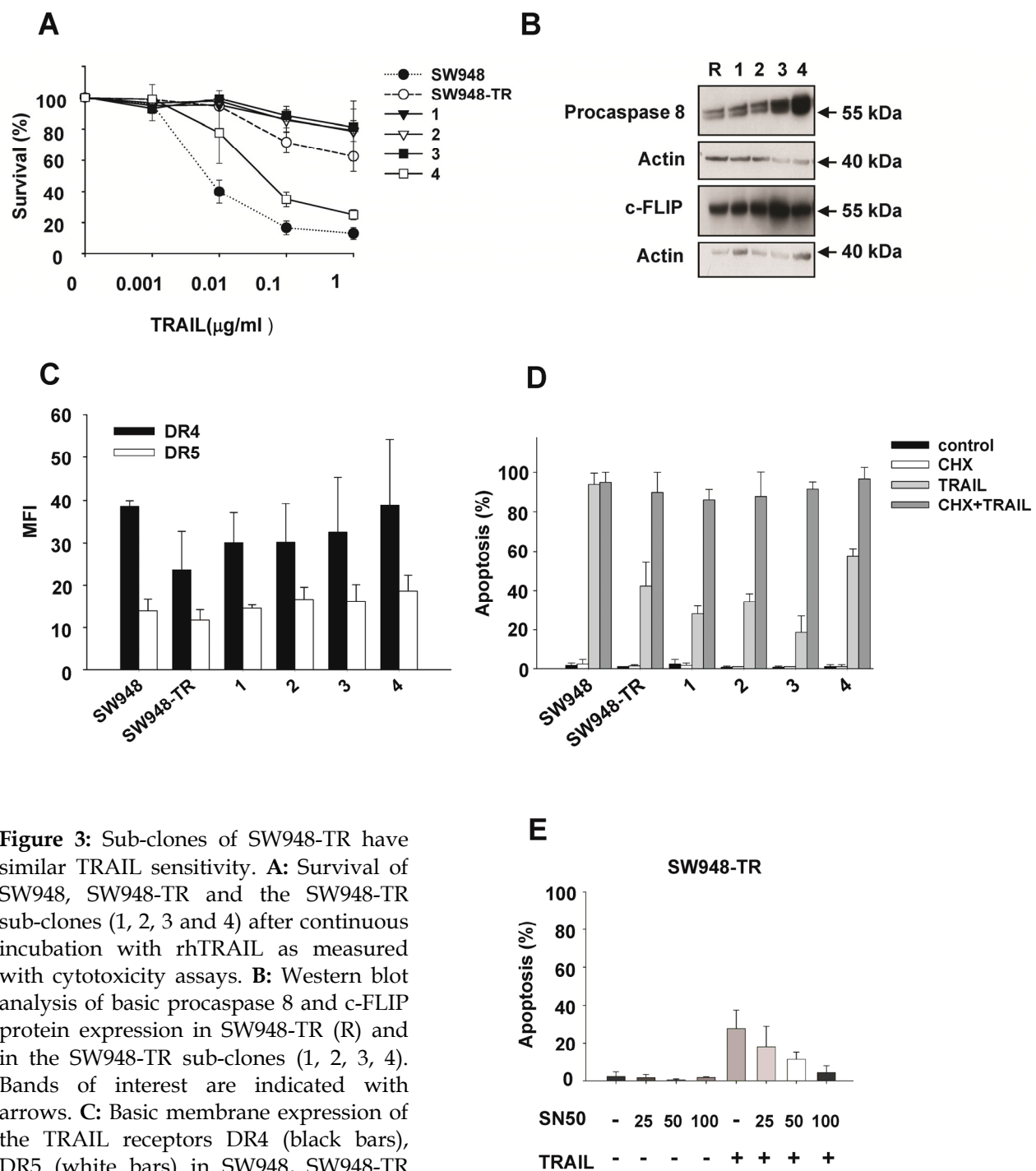


Figure 3: Sub-clones of SW948-TR have similar TRAIL sensitivity. **A:** Survival of SW948, SW948-TR and the SW948-TR sub-clones (1, 2, 3 and 4) after continuous incubation with rhTRAIL as measured with cytotoxicity assays. **B:** Western blot analysis of basic procaspase 8 and c-FLIP protein expression in SW948-TR (R) and in the SW948-TR sub-clones (1, 2, 3, 4). Bands of interest are indicated with arrows. **C:** Basic membrane expression of the TRAIL receptors DR4 (black bars), DR5 (white bars) in SW948, SW948-TR and in the sub-clones of SW948-TR (clones 1, 2, 3, 4). Expression was detected as mean fluorescence intensity (MFI) of the whole cell population. **D:** Apoptosis induced in SW948, SW948-TR and in the sub-clones of SW948-TR (clones 1, 2, 3, 4). Cells were plated as control (black bars), exposed to 5 μg/ml CHX (white bars), 0.1 μg/ml rhTRAIL (light grey bars) or pretreated with 5 μg/ml cycloheximide for 1 h followed by 5 h incubation with 0.1 μg/ml rhTRAIL (dark grey bars). **E:** The NFκB inhibitor SN-50 does not sensitize SW948-TR to rhTRAIL-induced apoptosis. Cells were pre-exposed to 25, 50 or 100 μg/ml SN-50 alone or in combination with 0.1 μg/ml rhTRAIL for 5 h.

The NF κ B pathway has sometimes been attributed pro- and anti-apoptotic properties as regard to TRAIL-sensitivity (20,21). SW948-TR cells were treated with SN-50, a cell permeable inhibitory peptide based on NF κ B p50, to investigate the role of NF κ B-activation in response to rhTRAIL (Figure 3E). Inhibition of NF κ B did not sensitize SW948-TR to rhTRAIL. Remarkably, SN-50 showed an apoptosis-inhibiting effect on the ~30% apoptotic cells detected after rhTRAIL exposure, suggesting potential pro-apoptotic effects of the NF κ B pathway in the rhTRAIL-sensitive fraction of the SW948-TR cell population.

The caspase 8/ c-FLIP ratio plays a role in the differential rhTRAIL-sensitivity of SW948 and SW948-TR cells

We previously reported that treating colon carcinoma cell lines with CHX strongly downregulated c-FLIP expression, notably in SW948 cells (15). CHX increased rhTRAIL-sensitivity (Figure 2A) and downregulated c-FLIP in SW948-TR cells (Figure 4A). The increase in Bid activation (Figure 2B) suggests that CHX primarily modulate rhTRAIL-induced apoptosis upstream of Bid by downregulating c-FLIP and thus facilitating caspase 8 cleavage. However, it cannot be ruled out that CHX inhibits expression of some additional anti-apoptotic proteins with a high turnover. To study the importance of c-FLIP downregulation by CHX in our TRAIL-resistant cell line, we downregulated c-FLIP levels in SW948-TR cells using short interference RNA technology. c-FLIP siRNA specifically downregulated c-FLIP levels compared to luciferase siRNA (Figure 4A). This demonstrates the importance of CHX-induced c-FLIP downregulation for the increase in rhTRAIL-sensitivity in SW948-TR. Downregulating c-FLIP expression sensitized SW948-TR cells to rhTRAIL-induced apoptosis (Figure 4C). Since SW948-TR exhibited lower levels of procaspase 8 rather than having higher levels of its inhibitor c-FLIP (Figure 1D), we tested the role played by caspase 8 levels in TRAIL sensitivity of the parental cell line. Caspase 8 levels were therefore downregulated using siRNA before TRAIL treatment in the parental cell line (Figure 4D). Cleavage of the executioner caspase substrate PARP was used to evaluate rhTRAIL-induced apoptosis in SW948 cells following caspase 8 downregulation. As seen in Figure 4E, treatment with 0.01 μ g/ml rhTRAIL induced cleavage of ~50% of the available full-length PARP into its active form (89 kD), both in control and in luciferase-transfected cells. At the same rhTRAIL concentration, caspase 8 downregulation almost completely prevented PARP

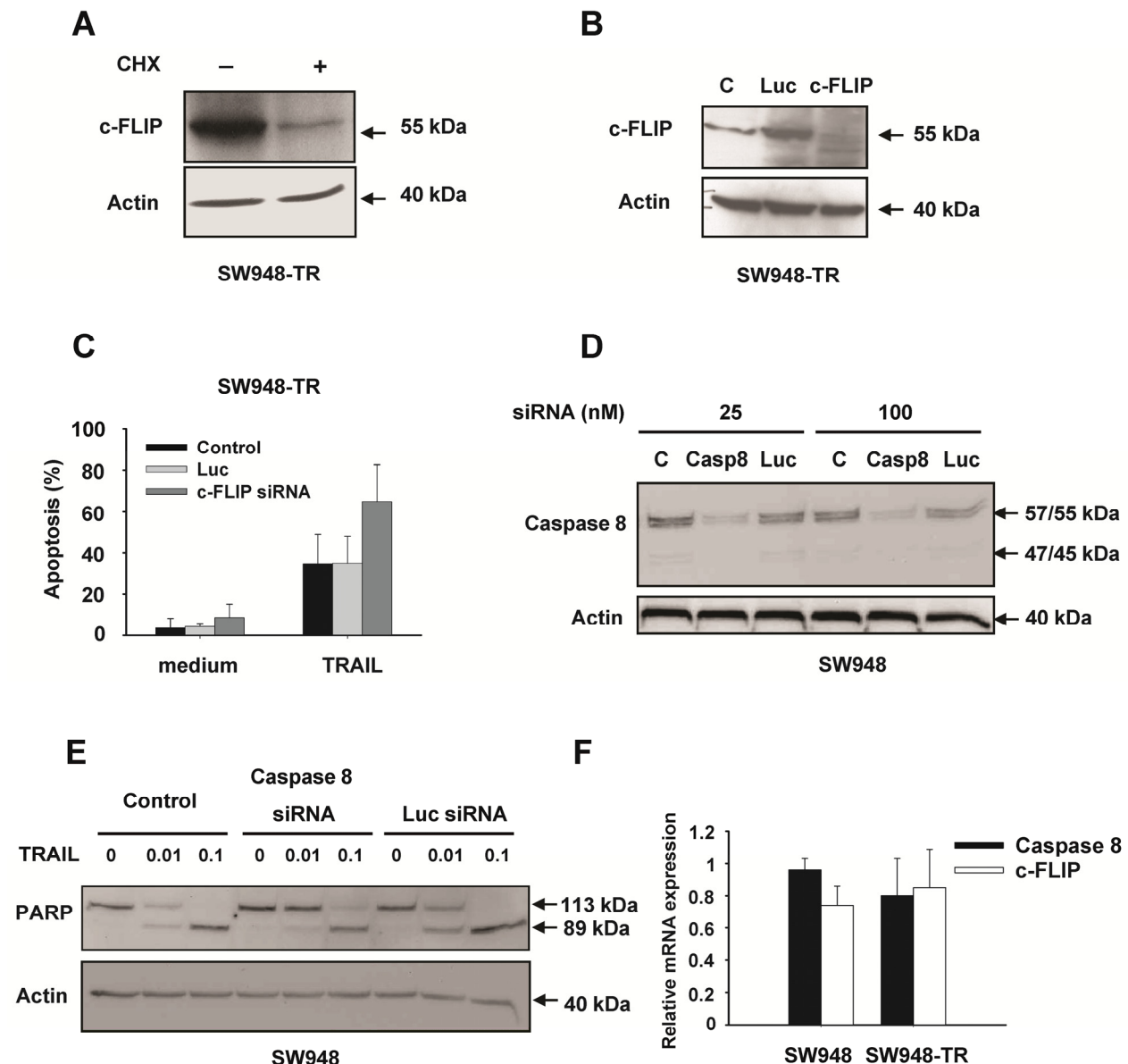


Figure 4: Caspase 8- and FLIP-levels are important regulator of TRAIL sensitivity in the SW948/SW948-TR cell lines. **A:** Western blot analysis of c-FLIP_L protein levels in SW948-TR cells after 24 h incubation with 5 μ g/ml CHX. **B:** SW948-TR cells were transfected with siRNA duplexes directed against the luciferase gene (Luc) as control siRNA or the c-FLIP gene. Western blot analysis showed down-regulation of c-FLIP by c-FLIP siRNAs. **C:** Apoptosis assay in siRNA transfected SW948-TR cells. Control cells and luciferase or c-FLIP siRNA transfected cells were plated and 48 h after transfection cells were incubated with rhTRAIL. After 4-5 h rhTRAIL incubation the percentage of apoptosis was determined ($p < 0.05$). **D:** Downregulation of caspase 8 by specific siRNAs in SW948-TR. Cells were transfected with 25-100 nM siRNA duplexes targeting luciferase (Luc) or the caspase 8 mRNA for 48 h before lysis and Western blot analysis. **E:** Effects of caspase 8 downregulation on PARP cleavage as determined by Western blot analysis. Cells were transfected with 66 nM siRNA for 48 h before treatment with rhTRAIL for 5 h before lysis. **F:** Relative caspase 8 and FLIP mRNA levels in SW948 and SW948-TR, as determined by real-time PCR.

cleavage in SW948. Thus, decreasing basal caspase 8 expression levels is sufficient to inhibit rhTRAIL-induced apoptosis in SW948 cells, despite the presence of caspase 10 (see Figure 1D). We did not completely prevent rhTRAIL-induced PARP cleavage in SW948 by inhibiting caspase 8 expression, in particular at higher concentrations of rhTRAIL (Figure 4E). This could be due to incomplete downregulation of caspase 8 (Figure 4D). Although procaspase 8 protein levels are lower in SW948-TR than in SW948 (Figure 1D), we now show that mRNA levels of caspase 8 (and c-FLIP) are similar between the two cell lines (Figure 4F), indicating that the decrease in caspase 8 levels in SW948-TR is due to decreased translation and/or decreased stability of the protein. Altogether, these results indicate that the caspase 8/c-FLIP ratio plays an important role for rhTRAIL sensitivity in SW948 and SW948-TR cells.

MG132 stabilized active caspase 8 protein levels and sensitized SW948-TR to rhTRAIL

As an alternative to c-FLIP downregulation using CHX, restoring caspase 8 levels may also restore rhTRAIL sensitivity in SW948-TR cells. To elucidate whether rhTRAIL resistance could be caused by increased proteasomal degradation of procaspase 8 levels, SW948-TR cells were treated with the proteasome inhibitor MG132 (Figure 5A). MG132 treatment did not increase procaspase 8 levels but induced a small increase in the 45/47 kDa intermediate product of caspase 8. No effect on full length c-FLIP levels was found. To importance of caspase 8 expression levels was studied in the rhTRAIL-treated surviving fraction of SW948-TR (Figure 5B, lane 2) which expressed low caspase 8 levels. Following rhTRAIL incubation for 5 h, apoptotic cells were washed away and the surviving fraction was incubated with MG132 for an additional 2-4 h (Figure 5B, lane 6-7). This induced high levels of both the intermediate product and the active form of caspase 8. The effect of MG132 treatment on c-FLIP levels was also assessed to provide an additional read-out for DISC activity (Figure 5B). In the surviving fraction, although some cleavage of c-FLIP was detected in presence of rhTRAIL, full-length c-FLIP was still available (Figure 5B, compare lane 2 to lane 3). While exposure to MG132 strongly increased intermediate and active caspase 8 levels in the surviving fraction, it only induced a slight augmentation in c-FLIP cleavage (Figure 5B, compare lanes 6-7 with lane 2). MG132 neither had effect on DR4 or DR5 protein levels nor on surface expression of these two receptors (data not shown). This indicates that the high levels of active caspase 8 found after MG132 treatment are not caused by an increase in death

receptor or procaspase 8 levels. Altogether, these experiments support a role for MG132 in stabilizing active caspase 8 generated at the DISC by TRAIL treatment.

Discussion

Previous research on long-term acquired TRAIL resistance mechanisms in colon carcinoma cell line reported the accumulation of Bax mutations *in vitro* and *in vivo* (9). Because most colorectal cancers are MMR-proficient, we analyzed the mechanisms of acquired rhTRAIL resistance in a MMR-proficient colon carcinoma cell line model. We now show that in these cells, resistance to rhTRAIL is caused by increased degradation of the pool of activated caspase 8. These results point toward preservation of the early TRAIL resistance mechanism and resembles results obtained in MMR-deficient TRAIL-resistant colon cancer cells obtained following four rounds of infection with TRAIL adenoviral vectors (22). In support of a generally critical involvement of active caspase destabilization mechanisms in TRAIL resistance, the stability of rhTRAIL-induced active caspase 3 (but not caspase 8) was found to be lower in a MMR-proficient ovarian cancer cell line exposed to rhTRAIL for 20 weeks.

The TRAIL-resistant SW948-TR cell line was established from the extremely TRAIL-sensitive SW948 colon carcinoma cell line after three months of continuous incubation with rhTRAIL. TRAIL receptor membrane expression was similar between the two cell lines. CHX sensitized SW948-TR cells to rhTRAIL as indicated by increased activation of both caspase 8 and Bid. Although SW948 and SW948-TR cells had similar c-FLIP protein levels, procaspase 8 levels were lower in SW948-TR, implying reduction of the caspase 8/c-FLIP ratio. We show that this ratio plays a crucial role in regulating TRAIL sensitivity in these cells. Downregulating c-FLIP protein levels with CHX increased rhTRAIL-sensitivity in SW948-TR cells. Treatment of SW948-TR with specific c-FLIP siRNA also sensitized SW948-TR cells to rhTRAIL, while downregulation of caspase 8 decreased rhTRAIL sensitivity in SW948. In rhTRAIL-treated SW948-TR cells, separation of the rhTRAIL-resistant surviving cell fraction from the apoptotic fraction showed that procaspase 8 levels were lower in the surviving fraction than in the apoptotic fraction or in the sensitive parental cell line. Additionally, only partial cleavage of full-length c-FLIP was found in the surviving cell fraction of SW948-TR, suggesting that the decrease in rhTRAIL-induced apoptosis was associated with lower

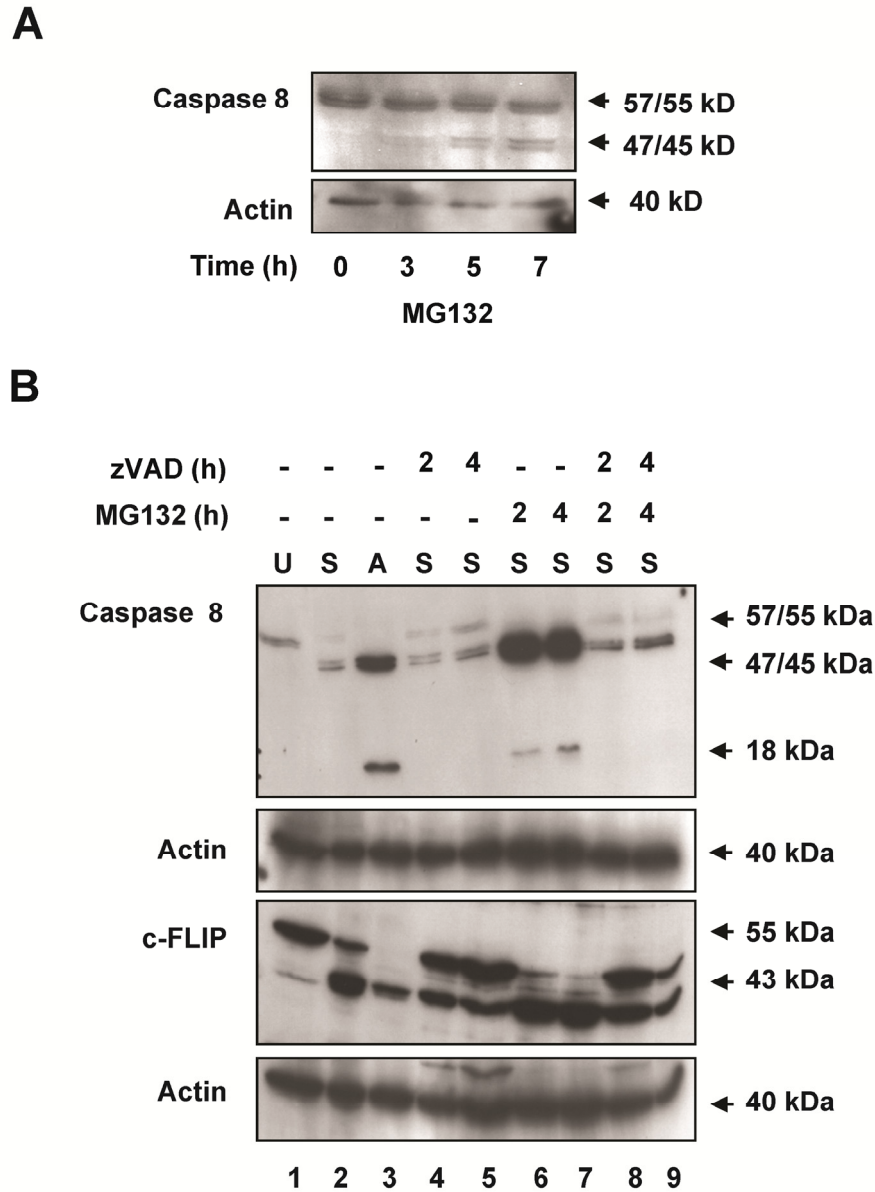


Figure 5: MG132 stabilizes active caspase 8 protein levels and sensitizes SW948-TR to TRAIL in a caspase-dependent manner. **A:** Western blot analysis of caspase 8 and c-FLIP protein levels in SW948-TR cells incubated with MG132 for 0-7 h. **B:** Western blot analysis of caspase 8 activation (casp 8) and c-FLIP processing in SW948-TR. After 5 h rhTRAIL incubation (0.1 $\mu\text{g}/\text{ml}$) the surviving cell fraction (S) was separated from the apoptotic cell fraction (A). U is untreated total cell population. Cells were then exposed to zVAD and or MG132 for 2 or 4 h.

DISC activity. Spencer *et al.* recently demonstrated that the so-called “fractional killing” of TRAIL was caused by cell-to-cell variability in protein expression due to noise in gene expression (23). The presence of a CD133 positive sub-population could also explain this

differential killing of rhTRAIL (24,25). Altogether, our results support a key role for the caspase 8/c-FLIP ratio in initiating the acquired rhTRAIL resistance of SW948-TR cells. Accordingly, several studies have shown that a low caspase 8/c-FLIP ratio contribute to poor TRAIL sensitivity in cancer cell lines (26-31). Previously, we have reported that both c-FLIP and caspase 8 expressions are increased in colon cancer compared to normal epithelium (32). These changes were accompanied by large variations in caspase 8/FLIP ratio between colon tumors, which suggest the presence of subgroups of patients with pro- and anti-apoptotic caspase 8/c-FLIP ratios.

We further investigated the decrease in caspase 8 in the TRAIL-resistant cell lines. Changes cell cycle distribution could not explain the differences in caspase 8 between SW948 and SW948-TR. Several studies observed downregulation of caspase 8 via hypermethylation of the caspase 8 promoter (33,34). Treatment of SW948-TR with the demethylating agent 5-aza-2 deoxycytidine had no effect on caspase 8 levels (results not shown). This finding is in line with the comparable mRNA levels present in SW948 and SW948-TR, indicating either decreased translation and/or decreased stability of caspase 8 in SW948-TR. As previously observed by Zhang *et al.*, proteasome inhibition could not restore procaspase 8 levels (22). SW948-TR cells could, however, be sensitized to rhTRAIL by MG132, in association with an increase in intermediate and active caspase 8 levels. These results suggest that proteasome inhibition prevented the degradation of the cleaved forms of caspase 8.

Little is known about the degradation mechanism(s) of procaspase 8 or active caspase 8. Bi-functional apoptosis regulator protein (BAR) was found to bind both forms of the protein. BAR possesses a RING domain with putative ubiquitin-protein isopeptide ligase-like activity. Stegh *et al.* speculated that active caspase 8 might be targeted for ubiquitination and proteasome-dependent degradation, although proteasome inhibitors failed to prevent caspase 8 degradation (35). Previously, a family of apoptotic inhibitors, the caspase 8 and 10 associated RING proteins (CARPs) has been identified (36). Binding of CARP1 and CARP2 negatively regulated death effector domain (DED) caspases by contributing to their ubiquitin-mediated proteolysis. Knockdown of CARPs did not result in an up-regulation of procaspase 8 but both TRAIL- and Fas-induced apoptosis were increased via enhanced caspase 8 processing. In *Drosophila*, MG132 treatment resulted in an accumulation of the processed form of the apical caspase DRONC but not of its full-length form (37). Recently, Jin *et al.* showed

that consecutively to TRAIL treatment, a cullin3 (CUL3)-based E3 ligase induces poly-ubiquitination of caspase 8 at the DISC, thereby causing caspase 8 aggregation and full activation of this protein (38). The authors also found that proteasome inhibition did not stabilize poly-ubiquitinated active caspase 8 in the moderately TRAIL-sensitive H460 cell line. In Bid-depleted Hela cells treated with CHX, active caspase 8 activity persisted for several hours after TRAIL treatment (39). Altogether, these results suggest that proteasomal degradation might be specific for the activated forms of caspase 8, and more prevalent in rhTRAIL-resistant cells.

A limited number of studies have looked at mechanisms of acquired TRAIL resistance by long-term culturing of cells in medium containing rhTRAIL. TRAIL resistance was associated with a change in TRAIL receptor membrane expression in osteosarcomas (40) and in melanomas (41). In another study TRAIL exposure selected for cells with Bax mutations in MMR-deficient colon carcinoma cell lines (9). The importance of caspase 8 in TRAIL sensitivity has been shown before in non-isogenic cell lines (23,26,27,42). Active caspase 8 degradation was involved in the TRAIL resistance of a MMR-deficient colon cancer sub-cell line obtained after four rounds of selection with adenoviral TRAIL vectors (22). We are, however, the first to show that increased degradation of active caspase 8 still plays a crucial role in rhTRAIL-resistant colon carcinoma cells obtained after long-term exposure to this agent.

In conclusion, acquired rhTRAIL-resistance in SW948-TR is caused by decreased basal procaspase 8/c-FLIP ratio and increased degradation of cleaved caspase 8 after rhTRAIL treatment. Modulating the procaspase 8/c-FLIP ratio using c-FLIP siRNA or by stabilizing active caspase 8 protein levels using proteasome inhibition can restore rhTRAIL sensitivity in MMR-proficient cells which have acquired rhTRAIL-resistance. These results may help overcoming TRAIL resistance using rational strategies with targeted drugs.

Acknowledgements

This work was supported by grant RUG 2000-2286 and grant RUG 2005-3361 from the Dutch Cancer Society and a grant from the Maurits en Anna de Kock Stichting.

References

1. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA and . Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995; 3: 673-682.
2. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A and Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996; 271: 12687-12690.
3. Koschny R, Walczak H and Ganten TM. The promise of TRAIL-potential and risks of a novel anticancer therapy. *J Mol Med* 2007; 85: 923-935.
4. Mahalingam D, Szegezdi E, Keane M, de Jong S and Samali A. TRAIL receptor signalling and modulation: Are we on the right TRAIL? *Cancer Treat Rev* 2009; 35: 280-288.
5. Oldenhuis CNAM, Stegehuis JH, Walenkamp AME, de Jong S and de Vries EGE. Targeting TRAIL death receptors. *Curr Opin Pharmacol* 2008; 8: 433-439.
6. Duiker EW, Mom CH, de Jong S, Willemse PH, Gietema JA, van der Zee AG and de Vries EGE. The clinical trail of TRAIL. *Eur J Cancer* 2006; 42: 2233-2240.
7. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z and Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999; 104: 155-162.
8. Falschlehner C, Emmerich CH, Gerlach B and Walczak H. TRAIL signalling: decisions between life and death. *Int J Biochem Cell Biol* 2007; 39: 1462-1475.
9. LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D and Ashkenazi A. Tumor-cell resistance to death receptor--induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nat Med* 2002; 8: 274-281.
10. Ionov Y, Peinado MA, Malkhosyan S, Shibata D and Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 1993; 363: 558-561.
11. Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC and Perucho M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997; 275: 967-969.
12. Peltomaki P. Deficient DNA mismatch repair: a common etiologic factor for colon cancer. *Hum Mol Genet* 2001; 10: 735-740.
13. Burns TF and El Deiry WS. Identification of inhibitors of TRAIL-induced death (ITIDs) in the TRAIL-sensitive colon carcinoma cell line SW480 using a genetic approach. *J Biol Chem* 2001; 276: 37879-37886.

14. Melcher R, Koehler S, Steinlein C, Schmid M, Mueller CR, Luehrs H, Menzel T, Scheppach W, Moerk H, Scheurlen M, Koehrl J and Al Taie O. Spectral karyotype analysis of colon cancer cell lines of the tumor suppressor and mutator pathway. *Cytogenet Genome Res* 2002; 98: 22-28.
15. van Geelen CMM, de Vries EGE, Le TKP, van Weeghel RP and de Jong S. Differential modulation of the TRAIL receptors and the CD95 receptor in colon carcinoma cell lines. *Br J Cancer* 2003; 89: 363-373.
16. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K and Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; 411: 494-498.
17. Lane D, Côté M, Grondin R, Couture M and Piché A. Acquired resistance to TRAIL-induced apoptosis in human ovarian cancer cells is conferred by increased turnover of mature caspase-3. *Mol Cancer Ther* 2006; 5: 509-521.
18. Lee TJ, Lee JT, Park JW and Kwon TK. Acquired TRAIL resistance in human breast cancer cells are caused by the sustained cFLIP(L) and XIAP protein levels and ERK activation. *Biochem Biophys Res Commun* 2006; 351: 1024-1030.
19. Barnhart BC, Alappat EC and Peter ME. The CD95 type I/type II model. *Semin Immunol* 2003; 15: 185-193.
20. Plantivaux A, Szegezdi E, Samali A and Egan L. Is there a role for nuclear factor kappaB in tumor necrosis factor-related apoptosis-inducing ligand resistance? *Ann N Y Acad Sci* 2009; 1171: 38-49.
21. Radhakrishnan SK and Kamalakaran S. Pro-apoptotic role of NF-kappaB: implications for cancer therapy. *Biochim Biophys Acta* 2006; 1766: 53-62.
22. Zhang L, Zhu H, Teraishi F, Davis JJ, Guo W, Fan Z and Fang B. Accelerated degradation of caspase-8 protein correlates with TRAIL resistance in a DLD1 human colon cancer cell line. *Neoplasia* 2005; 7: 594-602.
23. Spencer SL, Gaudet S, Albeck JG, Burke JM and Sorger PK. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. *Nature* 2009; 459: 428-432.
24. Sussman RT, Ricci MS, Hart LS, Sun SY and El Deiry WS. Chemotherapy-resistant side-population of colon cancer cells has a higher sensitivity to TRAIL than the non-SP, a higher expression of c-Myc and TRAIL-receptor DR4. *Cancer Biol Ther* 2007; 6: 1490-1495.
25. Zabalova R, Stantic M, Prokopova K, Dong LF and Neuzil J. Cancer cells with high expression of CD133 exert FLIP upregulation and resistance to TRAIL-induced apoptosis. *Biofactors* 2009; 34: 231-235.
26. Harper N, Farrow SN, Kaptein A, Cohen GM and MacFarlane M. Modulation of tumor necrosis factor apoptosis-inducing ligand- induced NF-kappa B activation by inhibition of apical caspases. *J Biol Chem* 2001; 276: 34743-34752.
27. Lippa MS, Strockbine LD, Le TT, Branstetter DG, Strathdee CA and Holland PM. Expression of anti-apoptotic factors modulates Apo2L/TRAIL resistance in colon carcinoma cells. *Apoptosis* 2007; 12: 1465-1478.

28. MacFarlane M, Harper N, Snowden RT, Dyer MJ, Barnett GA, Pringle JH and Cohen GM. Mechanisms of resistance to TRAIL-induced apoptosis in primary B cell chronic lymphocytic leukaemia. *Oncogene* 2002; 21: 6809-6818.
29. MacFarlane M. TRAIL-induced signalling and apoptosis. *Toxicol Lett* 2003; 139: 89-97.
30. Ganten TM, Haas TL, Sykora J, Stahl H, Sprick MR, Fas SC, Krueger A, Weigand MA, Grosse-Wilde A, Stremmel W, Krammer PH and Walczak H. Enhanced caspase-8 recruitment to and activation at the DISC is critical for sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by chemotherapeutic drugs. *Cell Death Differ* 2004; 11 Suppl 1: S86-S96.
31. Pennarun B, Meijer A, de Vries EGE, Kleibeuker JH, Kruyt FA and de Jong S. Playing the DISC: Turning on TRAIL death receptor-mediated apoptosis in cancer. *BBA-Rev Cancer* 2010; 1805: 123-140.
32. Heijink DM, Kleibeuker JH, Jalving M, Boersma-van Ek W, Koornstra JJ, Wesseling J and de Jong S. Independent induction of caspase-8 and cFLIP expression during colorectal carcinogenesis in sporadic and HNPCC adenomas and carcinomas. *Cell Oncol* 2007; 29: 409-419.
33. Fulda S, Kufer MU, Meyer E, van Valen F, Dockhorn-Dworniczak B and Debatin KM. Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. *Oncogene* 2001; 20: 5865-5877.
34. Harada K, Toyooka S, Shivapurkar N, Maitra A, Reddy JL, Matta H, Miyajima K, Timmons CF, Tomlinson GE, Mastrangelo D, Hay RJ, Chaudhary PM and Gazdar AF. Deregulation of caspase 8 and 10 expression in pediatric tumors and cell lines. *Cancer Res* 2002; 62: 5897-5901.
35. Stegh AH, Barnhart BC, Volkland J, Algeciras-Schimmich A, Ke N, Reed JC and Peter ME. Inactivation of caspase-8 on mitochondria of Bcl-xL-expressing MCF7-Fas cells: role for the bifunctional apoptosis regulator protein. *J Biol Chem* 2002; 277: 4351-4360.
36. McDonald ER, III and El Deiry WS. Suppression of caspase-8- and -10-associated RING proteins results in sensitization to death ligands and inhibition of tumor cell growth. *Proc Natl Acad Sci U S A* 2004; 101: 6170-6175.
37. Muro I, Hay BA and Clem RJ. The Drosophila DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J Biol Chem* 2002; 277: 49644-49650.
38. Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lill JR and Ashkenazi A. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell* 2009; 137: 721-735.
39. Hellwig C, Kohler B, Lehtivarjo A, Dussmann H, Courtney M, Prehn J and Rehm M. Real time analysis of tumor necrosis factor-related apoptosis-inducing ligand/cycloheximide-induced caspase activities during apoptosis initiation. *J Biol Chem* 2008; 283: 21676-21685.
40. Bouralexis S, Findlay DM, Atkins GJ, Labrinidis A, Hay S and Evdokiou A. Progressive resistance of BTK-143 osteosarcoma cells to Apo2L/TRAIL-induced apoptosis is mediated by acquisition of DcR2/TRAIL-R4 expression: resensitisation with chemotherapy. *Br J Cancer* 2003; 89: 206-214.

41. Nguyen T, Zhang XD and Hersey P. Relative resistance of fresh isolates of melanoma to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. *Clin Cancer Res* 2001; 7: 966s-973s.
42. Teitz T, Lahti JM and Kidd VJ. Aggressive childhood neuroblastomas do not express caspase-8: an important component of programmed cell death. *J Mol Med* 2001; 79: 428-436.

Modulation of TRAIL resistance in colon carcinoma cells: Different contribution of DR4 and DR5

Caroline M.M. van Geelen^{1#}

Bodvaël Pennarun^{1,2#}

Phuong T.K. Le¹

Elisabeth G.E. de Vries¹

Steven de Jong¹

both authors contributed equally to this work

Departments of ¹Medical Oncology, ²Gastroenterology,
University Medical Center Groningen,
University of Groningen, Groningen, the Netherlands

Submitted for publication

Abstract

RhTRAIL is a therapeutic agent, derived from the TRAIL cytokine, which induces apoptosis in cancer cells by activating the membrane receptors DR4 and DR5. Here, we investigated each receptor's contribution to rhTRAIL-sensitivity and rhTRAIL-resistance and assessed whether anti-DR4 or anti-DR5 agonistic antibodies could circumvent rhTRAIL resistance. Our study was performed in an isogenic model comprised of the SW948 human colon carcinoma cell line and its rhTRAIL-resistant sub-line SW948-TR. SW948 cells were sensitive to all three of the DR-targeting agents tested, although the anti-DR5 antibody induced only weak caspase 8 cleavage and low apoptosis levels. Nonetheless, anti-DR4 and anti-DR5 antibodies induced equivalent DISC formation and caspase 8 cleavage at the level of their individual receptors, indicating that further caspase 8 processing is impaired upon stimulation of DR5. SW948-TR cells were resistant to all DR-targeting agents due to their lower caspase 8 expression levels. Caspase 8 levels were restored by MG-132 or IFN- γ pre-treatment, and sensitivity to rhTRAIL and the anti-DR4 antibody was increased. Surprisingly, MG-132 also enhanced DR5-mediated apoptosis. These results highlight a critical difference between DR4 and DR5 apoptotic signaling modulation, with possible implications for future combinatorial regimens.

Introduction

Tumor necrosis factor related apoptosis inducing ligand is a member of the tumor necrosis factor (TNF) superfamily. Recombinant human TRAIL (rhTRAIL) is currently drawing attention in the field of cancer therapy because of its specificity for inducing apoptosis in tumor cells. Five TRAIL-receptors have been identified. DR4 and DR5 can transduce the apoptotic signal, whereas decoy receptor (DcR1), decoy receptor 2 (DcR2) and osteoprotegerin (OPG) presumably serve as decoy receptors, inhibiting TRAIL-mediated apoptosis (1,2). Administration of rhTRAIL in tumor-bearing animals induces significant tumor regression without systemic toxicity (3,4). Furthermore, rhTRAIL in combination with chemotherapy or radiotherapy greatly enhances anti-tumor efficacy both *in vitro* and *in vivo* (5-8).

The TRAIL apoptosis pathway can also be initiated by death receptor (DR)-specific agonistic antibodies. These anti-DR4 and anti-DR5 monoclonal antibodies, either used alone or in combination with chemotherapy (or irradiation), induce apoptosis in tumor cells *in vitro* and *in vivo* (9-12). Thus, both rhTRAIL and agonistic antibodies demonstrate interesting preclinical anti-tumor properties. A phase I clinical study with rhTRAIL has been initiated (13). Several phase I-II clinical studies with agonistic anti-DR4 antibody and a phase I study with agonistic anti-DR5 antibody have been performed (2,14,15). However, as rhTRAIL and DR-agonistic antibodies differently stimulate the apoptosis signaling cascade, drug-specific effects in the treatment of cancer patients are expected (16-18). rhTRAIL, which can bind to DR4 and DR5 but also to the decoy receptors, triggers cross-linking of these receptors into homo- and/or heterotrimers (19,20). In contrast, agonistic anti-DR4 or anti-DR5 antibodies have been suggested to trigger formation of multimeric complexes consisting of only one specific receptor, consequently bypassing the decoy receptors (21,22).

Not all tumor cells are sensitive to rhTRAIL, since intrinsic or acquired resistance to this ligand can exist. Very little is known about potential DR agonist-specific properties on downstream signaling pathways activation (e.g. NF κ B) and on rhTRAIL-resistance, but rhTRAIL and agonistic anti-DR5 antibodies exhibit different ability to induce the conformational changes of DR5 required to allow FADD recruitment (23).

The cytokine IFN- γ and proteasome inhibitors modulate several components of the apoptotic signaling pathway involved in TRAIL resistance (24-26). Combinations of

these drugs with TRAIL or death receptor agonist antibody can enhance TRAIL-induced apoptosis and overcome TRAIL-resistance in tumor cells (27-32). However, potential receptor specific effects of rhTRAIL resistance acquisition have not been investigated before. This is of major interest, as it is not yet established whether anti-DR4 or anti-DR5 antibodies or rhTRAIL have superior anti-tumor efficacy in the clinic. In addition, the biomarkers that should be used to select patients for the different anti-DR4 and anti-DR5 therapies are still unknown.

In the present study we used agonistic monoclonal antibodies to separately evaluate the roles of DR4 and DR5 in rhTRAIL-sensitivity and TRAIL-resistance. We compared rhTRAIL-induced apoptosis with agonistic DR4 and DR5 antibody-induced apoptosis in an rhTRAIL-sensitive human colon cancer cell line and its rhTRAIL-resistant sub-line. Furthermore we analyzed whether modulation of agonistic anti-DR4 and anti-DR5 induced apoptosis using IFN- γ or a proteasome inhibitor was comparable to modulation of rhTRAIL-induced apoptosis in these cell lines.

Materials and Methods

Reagents

RPMI 1640 medium was obtained from Life Technologies (Breda, The Netherlands) and fetal calf serum (FCS) from Bodinco BV (Alkmaar, the Netherlands). 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT)-solution and CHX were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). rhTRAIL was produced non-commercially in cooperation with IQ-Corporation (Groningen, the Netherlands) following a protocol described earlier (33). The agonistic anti-DR4 (HGS-ETR1) and anti-DR5 (HGS-TR2J) antibodies were a gift from Human Genome Sciences (HGS, Rockville, MD, USA). The inhibiting anti-DR4 (HS 101) and anti-DR5 (HS 201) antibodies were purchased from Alexis (10 P's BVBA, Breda, the Netherlands). The proteasome inhibitor MG-132 was obtained from Calbiochem (Breda, the Netherlands) and IFN- γ was purchased from Roche Diagnostics (Mannheim, Germany). The TRAIL receptor antibodies used for flow cytometry were obtained from Immunex Corporation (Seattle, WA, USA).

Cell lines

The rhTRAIL-sensitive colon carcinoma SW948 cell line (34) was cultured as described previously (35). In order to develop a rhTRAIL-resistant sub-line, SW948 was first exposed to 1.0 µg/ml rhTRAIL and incubated at 37 °C. After 14 days resistant cells were cultured twice a week in the presence of 2.5 µg/ml rhTRAIL. 3 months later the stable rhTRAIL-resistant cell line SW948-TR was obtained. In the absence of rhTRAIL in the culture medium SW948-TR remains resistant to rhTRAIL for at least 75 passages. This cell line is at least 140 fold more resistant to rhTRAIL than the sensitive parental cell line, as determined from the IC₅₀ at 96 h, and is similarly cultured.

Co-Immunoprecipitation of TRAIL-, DR4- or DR5-DISC

DISC immunoprecipitation after TRAIL-receptor ligation was performed according to Bodmer *et al.* (36) with some modifications. Briefly, 50.10⁶ cells per condition were grown, harvested and collected by centrifugation. The cell pellet was resuspended in 1 ml pre-warmed medium and the tube was placed in a 37 °C incubator. Recombinant human soluble flag-tagged TRAIL and anti-Flag monoclonal antibody M2 were premixed for 15-30 min on ice. Cells were stimulated in a final volume of 1 ml with 500 ng/ml Flag-tagged TRAIL and 1.5 µg/ml M2. In unstimulated cells the Flag-tagged TRAIL and M2 premix were added after lysis to immunoprecipitate non-stimulated TRAIL-receptors. Cell suspensions were incubated for 30 min at 37 °C, and the reaction was stopped by the addition of 10 ml ice-cold phosphate buffered saline (6.4 mM Na₂HPO₄; 1.5 mM KH₂PO₄; 0.14 mM NaCl; 2.7 mM KCl; pH=7.2). The cells were immediately washed with ice-cold PBS and lysed in 1 ml lysis buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) with complete protease inhibitors (Roche Diagnostics, Almere, the Netherlands) for 15 min on ice. After centrifugation (2,500 × g) at 4 °C for 10 min, the lysates were pre-cleared with 20 µl Sepharose-6B (Pharmacia, Uppsala, Sweden) for 2 h at 4 °C and immunoprecipitated with 30 µl protein-A sepharose beads for 4 h-overnight at 4 °C. Beads were washed three times with 1.5 ml lysis buffer, resuspended in standard Western blot sample buffer, and boiled for 5 min. Immunoprecipitated proteins were separated with SDS-PAGE. Western blot analysis for FADD, caspase 8, c-FLIP, DR4, DR5 and TRAIL was performed as described in the SDS-polyacrylamide gel electrophoresis and Western blotting section. Goat HRP-conjugated secondary antibody

specific for mouse IgG₁ and donkey-anti-goat-HRP were used for the detection of caspase 8, FADD, c-FLIP or DR4.

DR4 and DR5-DISC co-immunoprecipitation with the HGS-ETR1 and HGS-TR2J antibodies were performed as described above, with some modifications. Briefly, 50.10⁶ cells per condition were grown in medium harvested and resuspended in fresh medium. Cells were stimulated with 5µg/ml HGS-ETR1 or HGS-TR2J in a final volume of 2 ml. The antibodies were added after cell lysis for the control treatment. Cell suspensions were incubated for 15 min at 37 °C, and the reaction was stopped by the addition of 10 ml ice-cold phosphate buffered saline (PBS). The cells were immediately washed with ice-cold PBS and lysed in 1 ml lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) with complete protease inhibitors (Roche Diagnostics, Almere, the Netherlands) for 30 min on ice. After centrifugation (12,000 x g) at 4 °C for 10 min, the lysates were pre-cleared with 20 µl Sepharose-6B (Pharmacia, Uppsala, Sweden) for 2 h at 4 °C and immunoprecipitated with 50 µl protein-G agarose beads (Roche Diagnostics, Mannheim, Germany) for 3 at 4 °C. Beads were washed two times with 1 ml lysis buffer and one time with PBS before resuspension in standard Western blot sample buffer and boiling for 5 min. Immunoprecipitated proteins were separated with SDS-PAGE. Western blot analysis for FADD and c-FLIP was performed as described below. DR4 and DR5 were detected using rabbit anti-DR4 and rabbit anti-DR5 from ProSci Inc. (Poway, CA, USA). Caspase 8 was detected with rabbit anti-caspase 8 (Abcam plc, Cambridge, UK). Goat anti-rabbit HRP-conjugated secondary antibody and rabbit-anti-mouse-HRP were used for the detection of DR4, DR5, FADD, caspase 8 or c-FLIP.

SDS-polyacrylamide gel electrophoresis and Western blotting

Protein lysate preparation and Western blot analysis were performed as described previously (35).

Apoptosis assay

Apoptosis was assessed using acridine orange (AO) staining, using a method described earlier (35).

Cytotoxicity assay

The microculture tetrazolium (MTT) assay was used to determine cytotoxicity. SW948 and SW948-TR cells were incubated in a total volume of 200 μ l. After an incubation period of 96 h, 20 μ l of 5 mg/ml MTT solution diluted in PBS was added for 3.75 h. Subsequently, plates were centrifuged and the supernatant aspirated. After dissolving the formazan crystals by adding dimethyl sulfoxide (Merck, Amsterdam, the Netherlands), plates were read immediately at 520 nm using a microtiter well spectrometer (Bio-Rad microplate reader, Bio-Rad laboratories BV, Veenendaal, the Netherlands). Controls consisted of media without cells. Cell survival was defined as the growth of treated cells compared to untreated cells. IC₅₀ was the concentration of drug inhibiting survival by 50%. Mean cytotoxicity was determined in three independent experiments where each condition was performed in quadruplicate.

Flow cytometry

Analysis of DR4 and DR5 membrane expression was performed as described earlier (35). For competition experiments with rhTRAIL, SW948 cells ($2 \cdot 10^6$ /condition) were harvested, resuspended in 2.5 ml ice cold PBS and incubated on ice with various amounts of rhTRAIL for 1 h. Cells were then washed twice with PBS before fluorescent staining for DR4 and DR5 as described above.

Results

TRAIL-resistance in SW948-TR can be partially explained by its inefficient TRAIL-DISC formation

Recently, we have developed a model for acquired rhTRAIL-resistance consisting of the rhTRAIL-sensitive human colon carcinoma cell line SW948 and its rhTRAIL-resistant sub-line SW948-TR. In the two cell lines, DR4 and DR5 were both expressed at the cell surface, while DR4 seemed more highly expressed (see Figure 1A).

We next investigated whether differences in DR functionality may be involved in the rhTRAIL-resistance of SW948-TR. TRAIL-induced DISC formation was studied. Figure 1B demonstrates that SW948-TR exhibited weaker DISC recruitment of caspase 8 compared to the TRAIL-sensitive cell line. In particular, lower amounts of the cleaved p43/41 form of caspase 8 were found. Other DISC proteins such as DR4 and DR5,

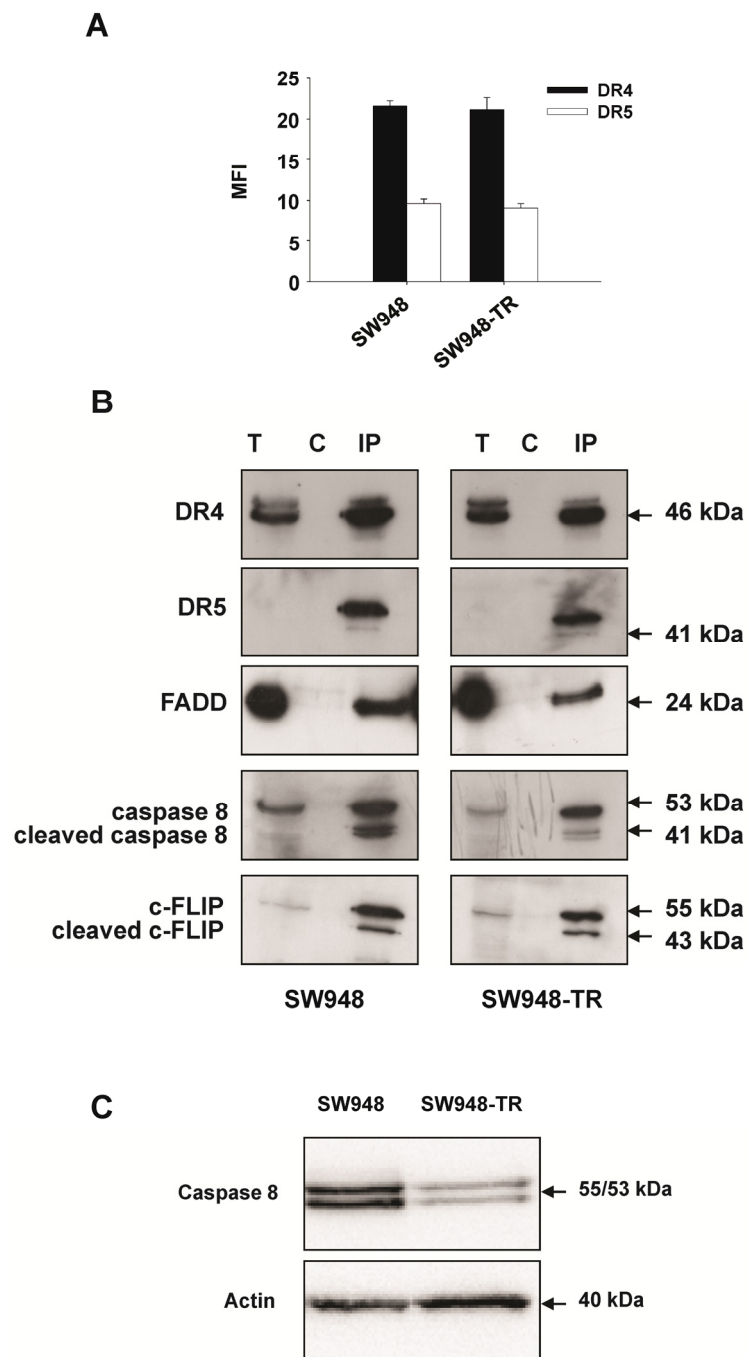


Figure 1. Inefficient DISC formation in SW948-TR cells. **A:** Membrane expression of the TRAIL receptors DR4 and DR5 in SW948 and SW948-TR cells as determined by flow cytometry. Values are expressed as the mean fluorescence intensity (MFI) and are mean \pm SE of at least three independent experiments. **B:** Analysis of the TRAIL-DISC in SW948 and SW948-TR. Cells were incubated for 30 min with Flag-tagged TRAIL and the assembled DISCs were immunoprecipitated and analyzed by Western blotting using antibodies to DR4, DR5, FADD, caspase 8 and c-FLIP. T = total cell lysates; C = control of immunoprecipitation, IP = immunoprecipitation (After a longer exposure DR5 bands were also detectable in the total cell lysates (T)). **C:** Western blot analysis comparing caspase 8 levels in SW948 and SW948-TR cells. One of at least three independent experiments is shown.

together with FADD and c-FLIP, were equally present in both cell lines. Looking for an explanation to the lower caspase 8 recruitment in the DISC of SW948-TR cells as compared to SW948 cells, we compared caspase 8 levels in whole cell lysates of both cell lines. As seen in Figure 1C, SW948 expressed lower amount of procaspase 8.

Specific blocking of the individual death receptors indicates that DR4 is more important than DR5 for rhTRAIL-induced apoptosis

To evaluate the contribution to apoptosis of either DR4 or DR5 in the sensitive and resistant cell line, antagonistic TRAIL receptor antibodies were used. In SW948, rhTRAIL-induced apoptosis was reduced by 60% using a blocking anti-DR4 antibody, while blocking DR5 only had a slight effect on rhTRAIL-induced apoptosis (Figure 2A). A competition experiment was performed in SW948 to look for possible mutations in the extracellular domain of DR4 or DR5 that would affect rhTRAIL-binding to DR4 or DR5. SW948 cells were pretreated with rhTRAIL and kept on ice to prevent receptor internalization, before detection of DR4 and DR5 using flow cytometry (Figure 2B). A similar decrease in detectable amounts of both death receptors for increasing concentrations of rhTRAIL was observed. Thus, we conclude that the binding of rhTRAIL is comparable for each DR in SW948 cells.

Following initial sensitization with the protein synthesis inhibitor CHX, SW948-TR cells were also exposed to rhTRAIL (Figure 2C). Apoptosis was decreased by 60% upon inhibition of rhTRAIL binding to DR4 with an anti-DR4 antibody. Inhibition with anti-DR5 antibody had almost no effect on rhTRAIL-induced apoptosis. Thus, blocking of DR4 and DR5 with antagonist antibodies revealed that DR4 was more important than DR5 for rhTRAIL-induced apoptosis in SW948 and SW948-TR. Surprisingly, in SW948 and SW948-TR the combination of both DR-antagonistic antibodies inhibited rhTRAIL-induced apoptosis even further, with 15.7% and 31.9% more, respectively, than anti-DR4 antibody alone. This suggests that although rhTRAIL mostly signals via DR4, both DR4 and DR5 might be important for maximum rhTRAIL-induced apoptosis signaling.

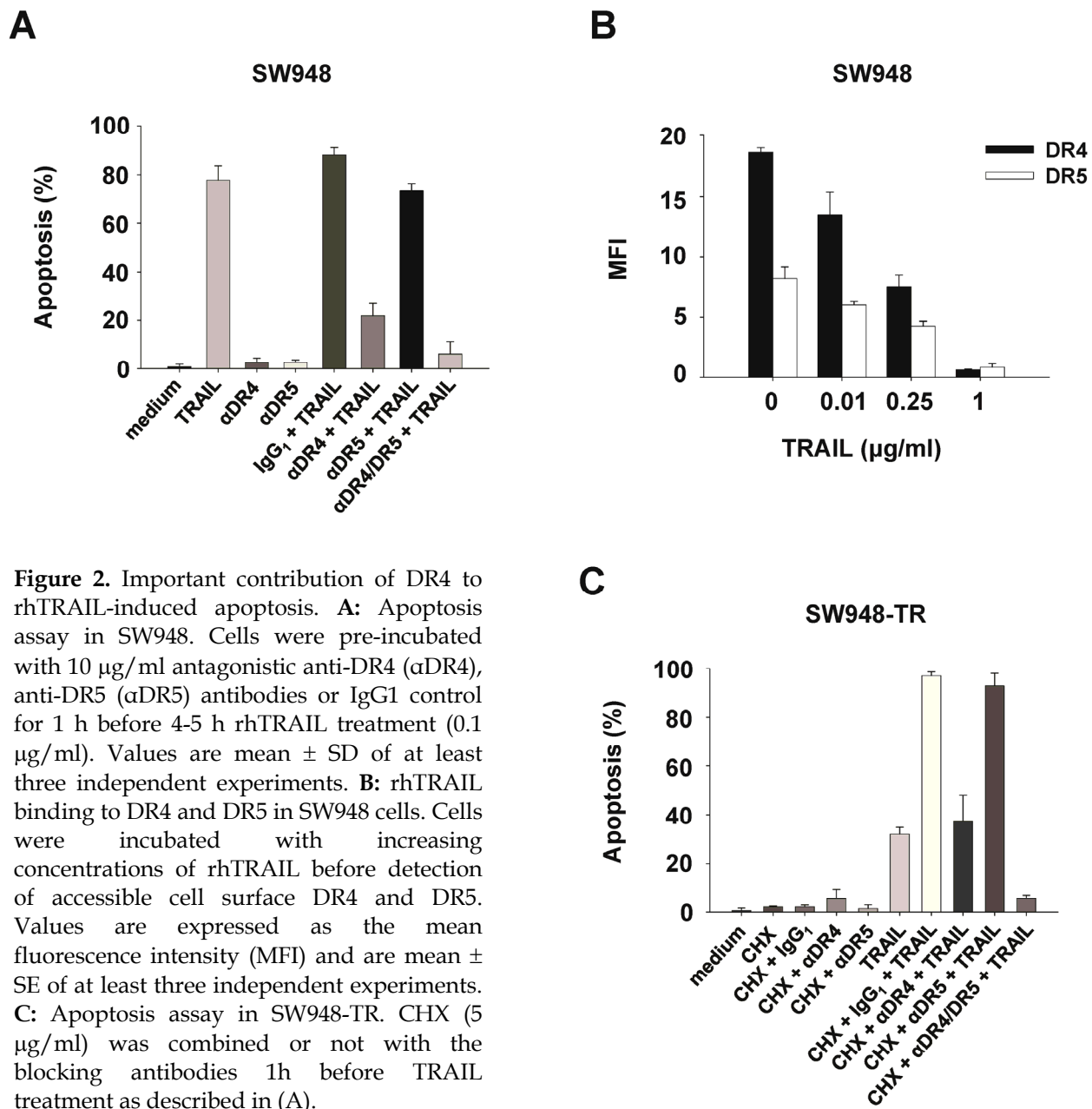


Figure 2. Important contribution of DR4 to rhTRAIL-induced apoptosis. **A:** Apoptosis assay in SW948. Cells were pre-incubated with 10 $\mu\text{g/ml}$ antagonistic anti-DR4 (αDR4), anti-DR5 (αDR5) antibodies or IgG1 control for 1 h before 4-5 h rhTRAIL treatment (0.1 $\mu\text{g/ml}$). Values are mean \pm SD of at least three independent experiments. **B:** rhTRAIL binding to DR4 and DR5 in SW948 cells. Cells were incubated with increasing concentrations of rhTRAIL before detection of accessible cell surface DR4 and DR5. Values are expressed as the mean fluorescence intensity (MFI) and are mean \pm SE of at least three independent experiments. **C:** Apoptosis assay in SW948-TR. CHX (5 $\mu\text{g/ml}$) was combined or not with the blocking antibodies 1h before TRAIL treatment as described in (A).

rhTRAIL sensitivity of each DR corresponds with sensitivity to agonistic anti-DR4 and anti-DR5 monoclonal antibodies

To gain more insight into the functionality of DR4 and DR5 in apoptosis signaling, the effects of agonistic monoclonal DR4 and DR5 antibodies on DR-mediated apoptosis were determined by survival assay. As shown in Figure 3A (left), the rhTRAIL-sensitive cell line SW948 was sensitive to both the HGS-ETR1 and HGS-TR2J antibody. The

agonistic anti-DR4 antibody was more effective than the agonistic anti-DR5 antibody, in particular at higher concentrations. SW948-TR cells were resistant to both HGS-ETR1 and HGS-TR2J (Figure 3A, right). These findings were confirmed by apoptosis measurements using AO staining (Figure 3B). Thus, the sensitivity to agonistic anti-DR4 or anti-DR5 antibodies of SW948 and SW948-TR cells seems to partially reflect the individual sensitivity to rhTRAIL of each receptor. Interestingly, the low sensitivity to the agonistic DR5 antibody in comparison to the agonistic DR4 antibody could be enhanced by CHX in SW948 (Figure 3B, left). CHX could also strongly sensitize SW948-TR cells to HGS-ETR1 and to a lesser extend to HGS-TR2J (Figure 3B, right).

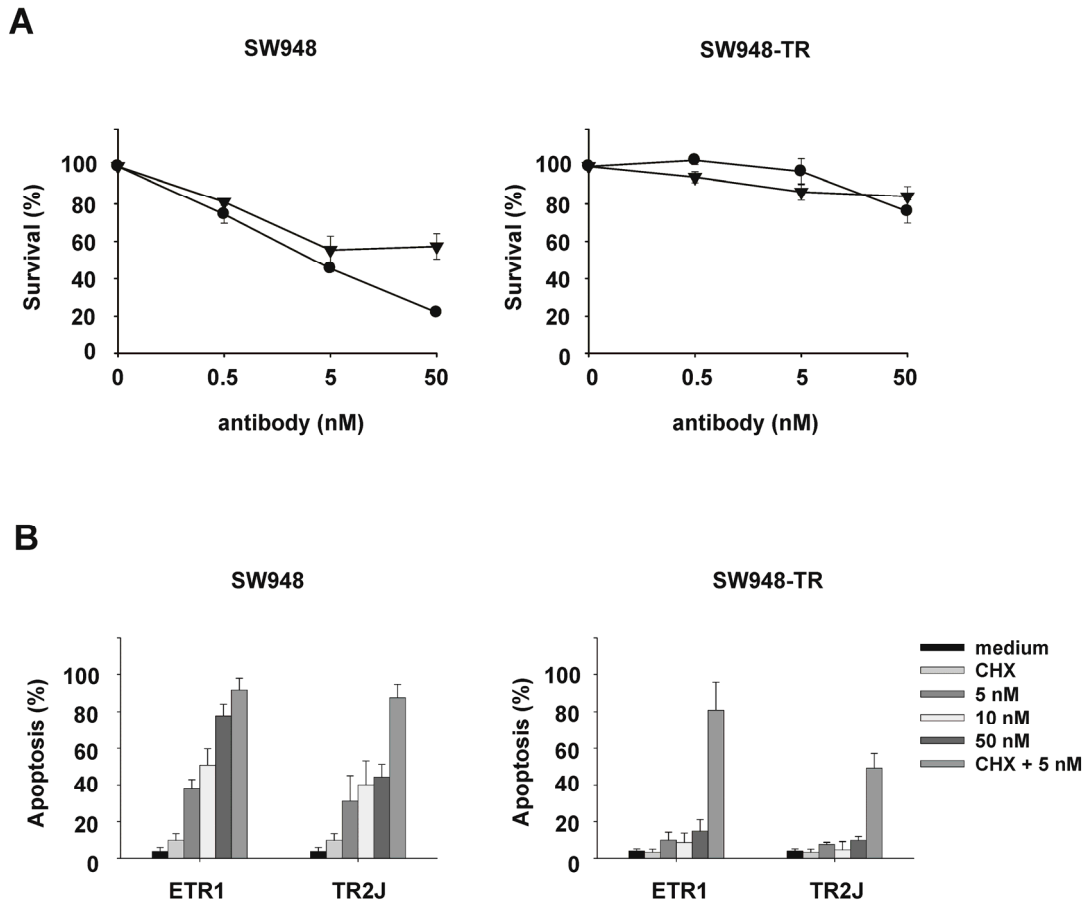


Figure 3. Sensitivity of SW948 and SW948-TR to HGS-ETR1 and HGS-TR2J antibodies. **A:** Survival (%) of SW948 and SW948-TR after continuous incubation with HGS-ETR1 (●) and HGS-TR2J (▲) as measured by cytotoxicity assays. **B:** Apoptosis assay in SW948 and SW948-TR. Cells were pre-incubated with 5 µg/ml CHX for 1 h before incubation with various concentrations of HGS-ETR1 or HGS-TR2J for 24 h.

Upon stimulation with agonistic antibodies, comparable DISC formation occurs at the level of DR4 and DR5 in SW948

We showed that DR4 was more competent at initiating apoptosis when compared to DR5 in SW948 cells. This was demonstrated by stimulating each receptor with rhTRAIL in combination with blocking antibodies, but also by stimulating each receptor with agonistic antibodies only. To investigate possible differences in DR4-DISC versus DR5-DISC formation, we stimulated SW948 cells with either HGS-ETR1 or HGS-TR2J, and individually co-immunoprecipitated DR4- and DR5-DISC (Figure 4A). Surprisingly, DISC formation was comparable for both stimuli. HGS-ETR1 could recruit DR4, FADD, caspase 8 and c-FLIP. Using an antibody directed against the N-terminal fragment of caspase 8, three forms of this protein were detected in the DR4-DISC: the full form, the intermediate p43/41 cleaved form and the p26/24 cleavage product. The latter indicates full cleavage of caspase 8 and thereby release of active caspase 8 from the complex (for comprehensive review see (37)). c-FLIP was mostly present in its intermediate form. HGS-TR2J recruited DR5 and similar amounts of FADD, c-FLIP and caspase 8 as compared to HGS-ETR1. Later time points displayed similar DISC content (data not shown). Caspase 8 cleavage was also investigated in whole cell lysates of SW948 cells treated with either DR-agonistic antibody (Figure 4B). Within 3 hours, HGS-ETR1 induced full cleavage of the available procaspase 8 into its intermediate and active form. Caspase 8 cleavage in cells stimulated with HGS-TR2J was only partial, as active caspase 8 could not be detected, while unprocessed procaspase 8 molecules were still present after 3 hours.

Resistance to rhTRAIL, HGS-ETR1 and HGS-TR2J can be overcome by the proteasome inhibitor MG-132

Several studies have demonstrated that proteasome inhibition can overcome rhTRAIL-resistance (24,38,39). Previously, we have observed that SW948-TR could be sensitized to rhTRAIL using the proteasome inhibitor MG-132 (unpublished observations). To investigate whether MG-132 sensitized cells in receptor-specific manner, SW948-TR cells were pre-incubated with this compound before treatment with rhTRAIL or agonistic anti-DR4 and -DR5 antibodies. HGS-ETR1- and HGS-TR2J-induced apoptosis was enhanced by approximately 50-60% following MG-132 treatment, which was similar to the observed enhancement of rhTRAIL-induced apoptosis (Figure 5A). As seen in Figure

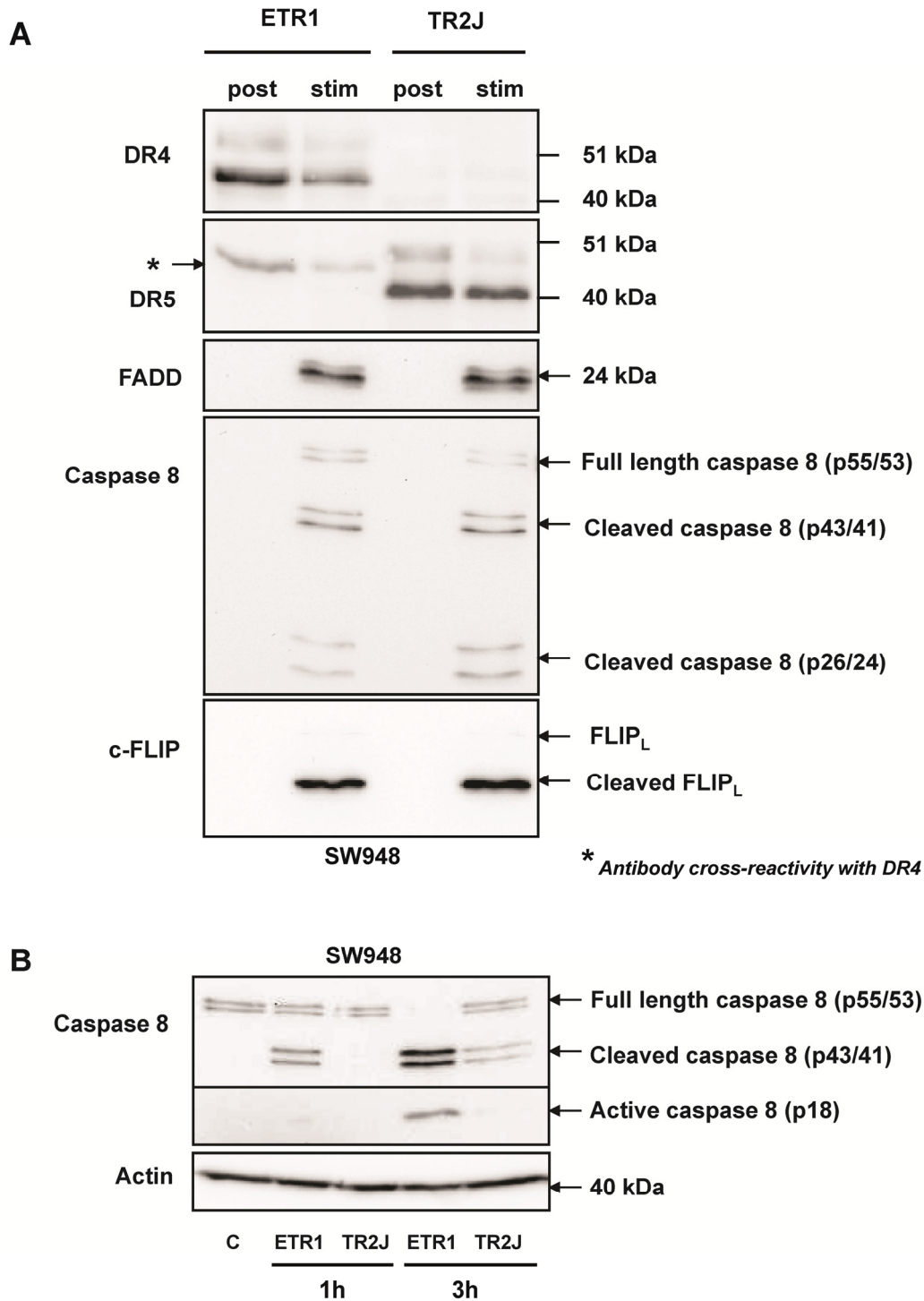


Figure 4: Equivalent DISC protein recruitment but not caspase 8 activation following HGS-ETR1 and HGS-TR2J treatment. **A:** Analysis of the DR4 and DR5-DISC in SW948. **A:** Cells were incubated for 15 min with HGS-ETR1 or HGS-TR2J before co-immunoprecipitation of the associated DISCs using protein G-agarose beads, and subsequent analysis by Western blotting using antibodies directed against DR4, DR5, FADD, caspase 8 and c-FLIP. Post = antibodies added after cell lysis; Stim = cells stimulated with the indicated antibody for 15 min. One representative of at least two independent experiments is shown. **B:** Time-dependent cleavage of pro-caspase 8 in SW948 cells treated with 50 nM HGS-ETR1 or HGS-TR2J.

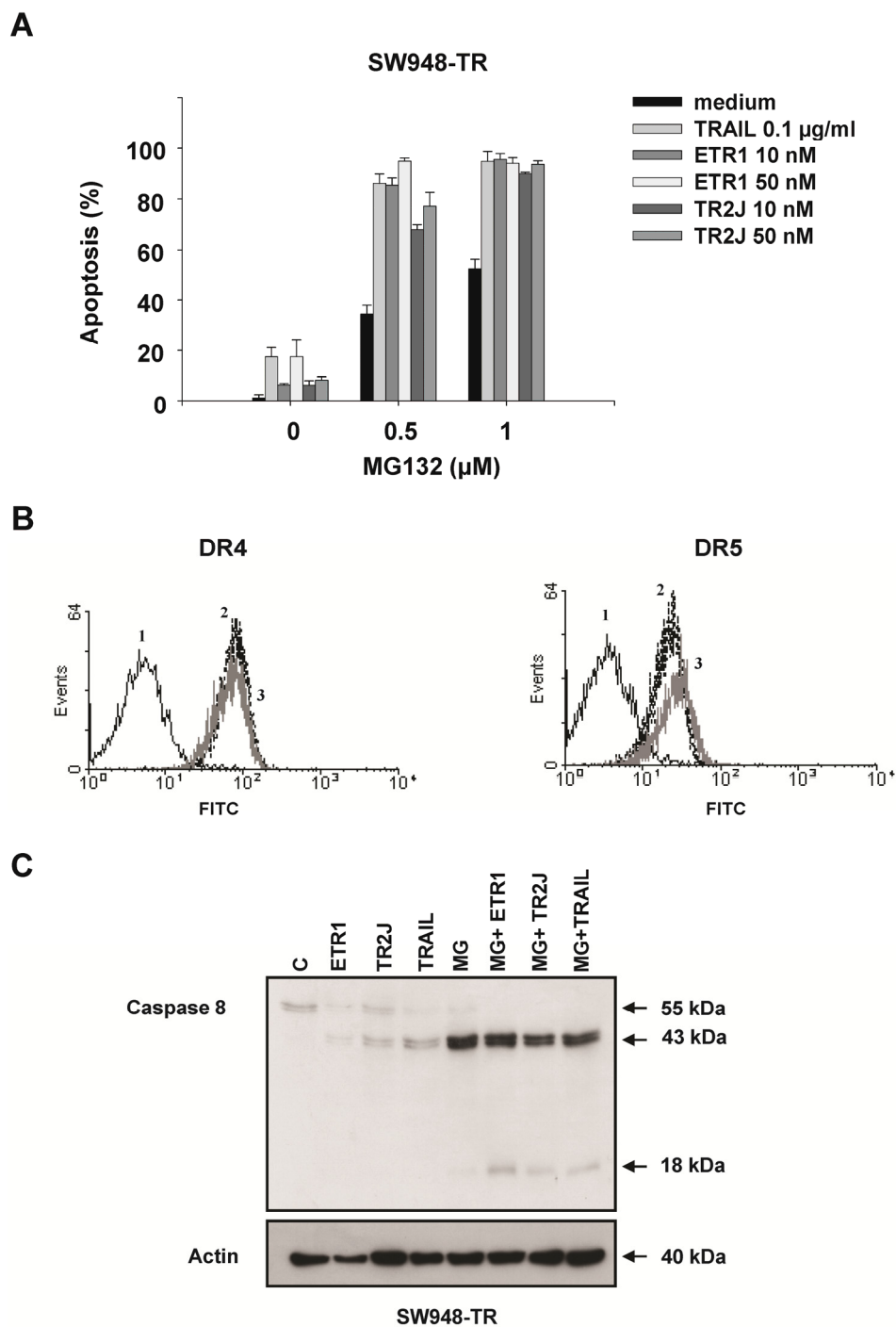


Figure 5: Sensitization of SW948-TR to rhTRAIL, HGS-ETR1 and HGS-TR2J by MG-132. **A:** Apoptosis assay in SW948-TR after 17 h of incubation with MG-132 in combination with rhTRAIL, HGS-ETR1 or HGS-TR2J. **B:** The effect of MG-132 on DR4 (left figure) and DR5 (right figure) membrane expression as determined by flow cytometry. Receptor expression was detected as the average antigenic density of the whole cell population and resulted in a peak shift to the right. (1 = control; 2 = basal DR4 or DR5 membrane expression level; 3 = DR4 or DR5 expression after exposure to 10 μ M MG-132 for 17 h). **C:** Western blot analysis of caspase 8 activation in SW948-TR after 17 h incubation with 1 μ M MG-132 in combination with rhTRAIL (0.1 μ g/ml), HGS-ETR1 or HGS-TR2J (50 nM).

5B, the effects were not the result of cell membrane DR4 (left figure) or DR5 (right figure) up-regulation by MG-132. Western blot analysis demonstrated that MG-132 increased the intermediate p43/41 cleavage product of caspase 8 (Figure 5C). The combination of MG-132 with either rhTRAIL, HGS-ETR1 or HGS-TR2J led to an almost identical processing of caspase 8 into its active caspase 8 subunit (p18).

IFN- γ sensitizes SW948-TR to rhTRAIL and HGS-ETR1 but not to HGS-TR2J

Our experiments with MG-132 suggested that TRAIL-sensitivity might be increased due to the enhancement of caspase 8 expression. Upregulation of caspase 8 by IFN- γ has been described as a mechanism of sensitization to rhTRAIL (40). Since reduced caspase 8 expression levels were detected in SW948-TR as compared to SW948 (Figure 1C), we hypothesized that IFN- γ might overcome rhTRAIL resistance in SW948-TR. Upon treatment with IFN- γ , caspase 8 protein expression was enhanced in a concentration-dependent manner, in both cell lines (Figure 6A). We excluded an effect of IFN- γ on cell surface DR expression levels in these cells, as no change in either DR4 or DR5 surface expression was observed following IFN- γ treatment (Figure 6B left and right, respectively). In a previous study we did not observe any additional effect of IFN- γ on rhTRAIL sensitivity in SW948, a cell line that is already extremely sensitive to rhTRAIL (35). Survival assays now showed that IFN- γ could sensitize SW948-TR to both rhTRAIL and HGS-ETR1 but not to HGS-TR2J (Figure 7A). Similar results were also seen in an apoptosis assay using AO staining (Figure 7B). Cell survival assays in SW948 indicated that IFN- γ enhanced the sensitivity to HGS-ETR1 but not to HGS-TR2J (results not shown). TRAIL receptor blocking antibodies were used to determine the relative contribution of each individual DR to IFN- γ induced sensitization to rhTRAIL in SW948-TR. The observed sensitization to rhTRAIL was DR4-specific, since the anti-DR4 blocking antibody reduced rhTRAIL-induced apoptosis by 50%, whereas the anti-DR5 blocking antibody only had a minor effect on apoptosis in SW948-TR cells pre-sensitized with IFN- γ (Figure 7C). Western blot analysis of caspase 8 cleavage was performed to gain further insights into the possible mechanism underlying IFN- γ -induced sensitization of DR-mediated apoptosis in SW948-TR cells (Figure 7D and E). IFN- γ elevated procaspase 8 levels and induced cleavage of procaspase 8 into the intermediate p43/41 product. The combination of IFN- γ with rhTRAIL or HGS-ETR1 induced cleavage of caspase 8 to its p18 active form. When cells were treated with IFN- γ and

HGS-TR2J, only the intermediate form of caspase 8 (p43/41) could be detected. These results contrasted with the most effective combination of HGS-TR2J with MG-132 (see Figure 5C). This suggests that only DR4-mediated apoptosis benefits from the increase in caspase 8 levels in IFN- γ pretreated cells.

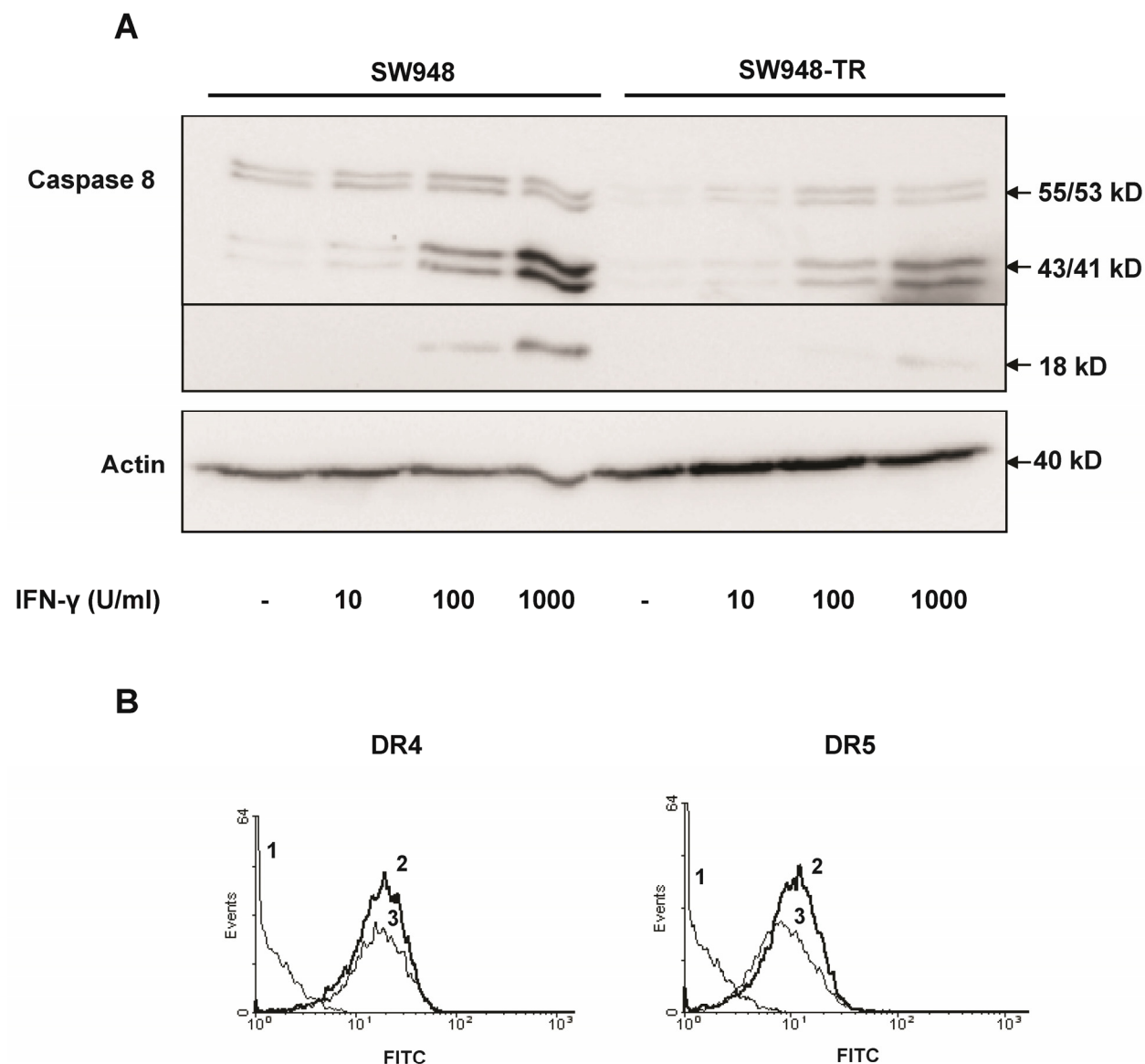


Figure 6: Increase in caspase 8 levels following treatment of SW948-TR with IFN- γ . **A:** Effects of 48 h incubation with increasing concentrations of IFN- γ on caspase 8 levels in SW948 and SW948-TR. **B:** Effects of IFN- γ on DR4 (left figure) and DR5 (right figure) membrane expression as determined by flow cytometry. Receptor expression was detected as the average antigenic density of the whole cell population and resulted in a peak shift to the right. (1 = control; 2 = basal DR4 or DR5 membrane expression level; 3 = DR4 or DR5 expression after exposure to 1000 units (U) /ml IFN- γ for 48 h).

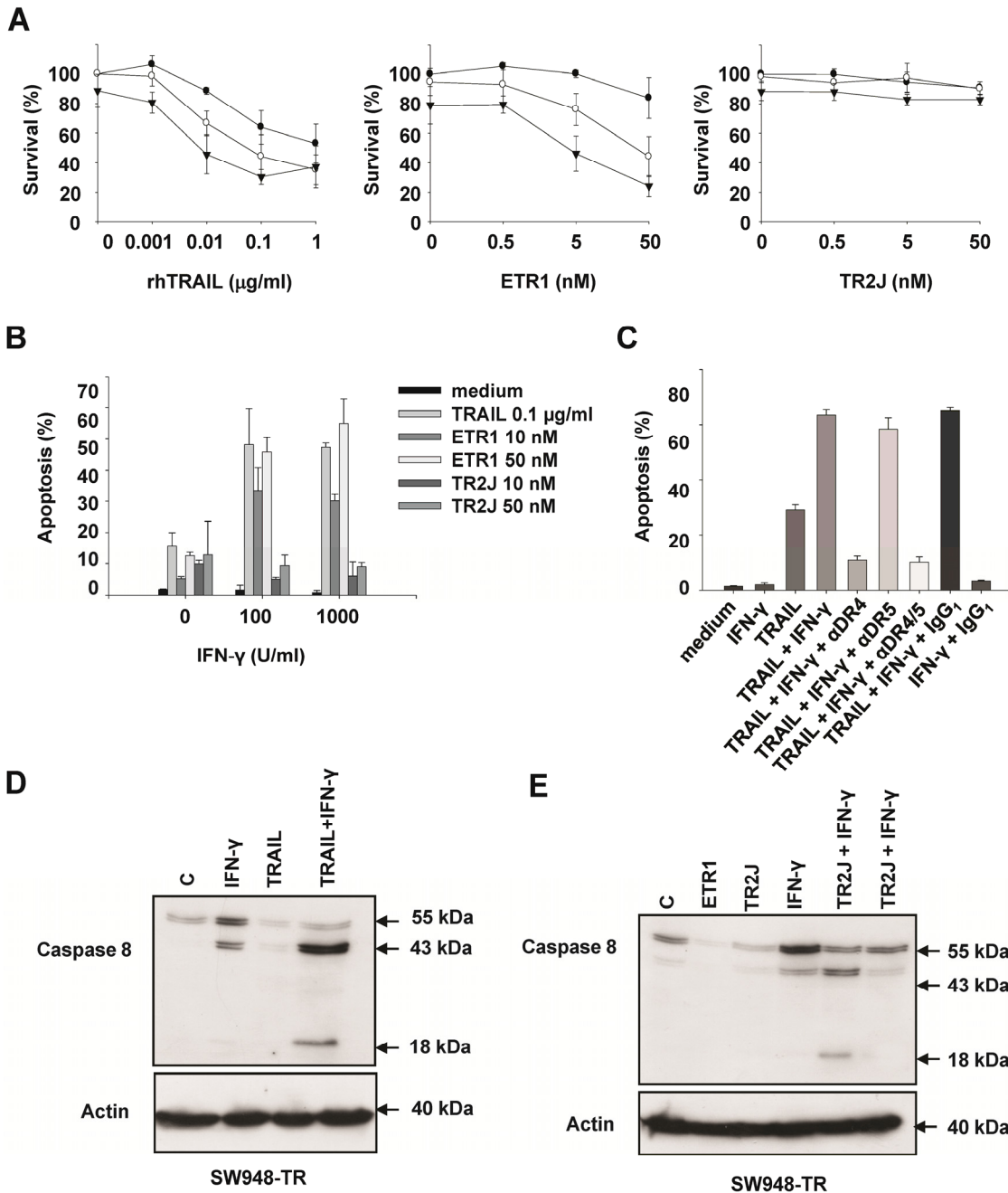


Figure 7: Stimulation of rhTRAIL and HGS-ETR1-induced but not HGS-TR2J-induced apoptosis by IFN-γ. **A:** Survival (%) of SW948-TR cells after continuous incubation with rhTRAIL, HGS-ETR1 or HGS-TR2J in combination with 0 (●), 10 (○), 1000 (▼) U/ml IFN-γ. **B:** Apoptosis assay of SW948-TR after 48 h incubation with rhTRAIL, HGS-ETR1 or HGS-TR2J in combination with various concentrations of IFN-γ. **C:** Apoptosis assay in SW948-TR after 48 h incubation with 1000 U/ml IFN-γ. Cells were then incubated with 10 μg/ml antagonistic anti-DR4 (αDR4), anti-DR5 (αDR5) antibodies, both (αDR4/5) or IgG1 control for 1 h before 4-5 h rhTRAIL treatment (0.1 μg/ml). **D:** Western blot analysis of caspase 8 activation in control cells (C), cells treated with IFN-γ or rhTRAIL (T), or the combination of both drugs. Cells were analyzed after 48 h of incubation with or without 1000 U/ml IFN-γ before 4-5 h treatment with rhTRAIL (0.1 μg/ml). **E:** Western blot analysis of caspase 8 activation in control cells (C), cells treated for 4-5h with HGS-ETR1 or HGS-TR2J (50 nM), or cells exposed to the agonistic antibodies following 48 h-incubation

Discussion

DISC formation is the first step toward apoptosis after engagement of DR4 and DR5 by their ligands. Not surprisingly, resistance to TRAIL-mediated apoptosis is often initiated at the DISC level (41). We found that upon stimulation with TRAIL, all canonical DISC proteins were recruited to DR4 and DR5 in SW948 and SW948-TR cells. In relation with the other DISC proteins, smaller amounts of cleaved caspase 8 were found in the TRAIL-DISC of SW948-TR cells as compared to SW948 cells. These results were in agreement with the lower basal caspase 8 protein levels found in SW948-TR. Altogether, these findings point toward less active recruitment and processing of caspase 8 in these cells. We also describe a DR5-specific mechanism of apoptosis resistance in our cell line model since DR4 stimulation achieves superior caspase 8 processing than DR5 despite equivalent initial DISC formation at the level of each receptor.

Antagonistic anti-DR4 or anti-DR5 antibodies were used to specifically block the function of their respective receptors during rhTRAIL-induced apoptotic signaling. These experiments proved that DR4 was more critical to rhTRAIL-induced apoptosis signaling than DR5, in both cell lines. It was previously reported that apoptosis induction in keratinocytes with leucine zipper TRAIL was also mainly mediated by DR4 (42). In contrast, Kelley *et al.* generated receptor selective mutants of TRAIL, with three to six ligand amino acid substitutions (17), and found DR5 to be more important for apoptotic signaling than DR4 in cancer cells expressing both receptor (including colon cancer cells). Van der Sloot *et al.* demonstrated that DR5-selective TRAIL variants did not induce apoptosis in cell lines mostly responsive to DR4 stimuli but greatly stimulated apoptosis in DR5-responsive cancer cell lines (43). Little is known about the origins of these discrepancies. Blocking of both DR4 and DR5 prevented apoptosis more efficiently than single blocking of DR4, suggesting that DR5 also contributes to rhTRAIL-induced apoptosis. While DR4 was more potent in transducing apoptosis, DR5 might, at least in our model, increase the overall apoptotic stimulus compared to DR4 stimulation alone.

We found that in SW948 and SW948-TR cells, sensitivity to HGS-ETR1 and HGS-TR2J antibodies mostly reflected the sensitivity to rhTRAIL of each receptor. Although the agonistic anti-DR4 antibody was more potent than its anti-DR5 counterpart, HGS-TR2J could induce apoptosis at some extent in SW948 cells, and also in SW948-TR

following pre-sensitization with CHX. This strongly suggests that DR5 is potentially functional in both cell lines. Georgakis *et al.* found that in primary non-Hodgkin's lymphoma samples, an anti-DR4 agonistic antibody was also more effective than an agonistic anti-DR5 antibody (16).

We investigated the ability of each receptor to initiate DISC formation and caspase 8 cleavage in SW948 by co-immunoprecipitating DR4- and DR5-DISC. To our surprise, similar DISC formation and caspase 8 cleavage were triggered at the level of both receptors, although caspase 8 processing was much higher in whole cell lysates of cells stimulated with HGS-ETR1 compared to HGS-TR2J. Resistance to DR5-mediated apoptosis begins after the initial caspase 8 cleavage, and could be due to a lower turn-over of procaspase 8 at the DISC. Data on such DISC protein turn-over following TRAIL receptor stimulation are limited, although studies by McDonald *et al.* and Jin *et al.* put forward the influence of caspase 8 ubiquitination on the cleavage of the available caspase 8 cellular pool (44,45). Because brief pre-incubation with CHX could also restore DR5-mediated caspase 8 cleavage and sensitivity, a short-lived protein might be involved in this process.

IFN- γ is known to increase caspase 8 expression and consequently sensitizes cancer cells, including colon cancer cells, to rhTRAIL (24,40,46-48). Our results slightly contrast with these findings, as upregulation of caspase 8 by IFN- γ did not enhance apoptosis induction in SW948 cells. Caspase 8 levels might not be a limiting factor in these cells since procaspase 8 recruitment to the DISC is first determined by the amount of FADD molecules available at the DISC (49). IFN- γ did, however, increase apoptosis induction by rhTRAIL and HGS-ETR1 in SW948-TR cells, which express lower caspase 8 levels than their parental cell line. In SW948-TR, IFN- γ induced a marked increase in both pro- and intermediate forms of caspase 8, which were cleaved to the active form upon DR4 stimulation. In contrast, DR5 stimulation failed to induce the appearance of active caspase 8. This was shown using DR-agonistic antibodies, but also rhTRAIL in combination with receptor-blocking antibodies.

Unlike IFN- γ , MG-132 markedly increased both DR4 and DR5-mediated sensitivities in SW948-TR cells. Another proteasome inhibitor, bortezomib, was previously reported to enhance the effect of agonistic anti-DR4 and agonistic anti-DR5 antibody in Hodgkin's disease cell lines (18,50). MG-132 could increase TRAIL-induced apoptosis in both Bax-deficient and proficient colon cancer cells, suggestive of a

sensitization mechanism independent of the mitochondrial pathway of apoptosis (24). DR4 and DR5 upregulation by proteasome inhibitors is thought a major factor contributing to TRAIL-induced apoptosis sensitization, although this hypothesis has been subject to an extensive debate (51). We found that upon MG-132 treatment, DR4-mediated sensitivity increased in absence of DR4 upregulation. The intermediate form of caspase 8 was upregulated by MG-132, an event we found sufficient to restore HGS-ETR1 or rhTRAIL sensitivity following IFN- γ treatment. Importantly, MG-132 also increased caspase 8 cleavage and enhanced DR5-mediated apoptosis. MG-132 has previously been shown to modulate DR5 expression (24,52). Our flow cytometry analysis only showed negligible DR5 upregulation by MG-132, which again points towards a mechanism of sensitization independent of this effect. MG-132 upregulated caspase 8 in SW948-TR, but we demonstrated using IFN- γ that restoring caspase 8 levels was not sufficient to increase DR5-mediated caspase 8 cleavage and apoptosis. Taken together, these results support the existence of additional sensitizing mechanisms of MG-132 at the level of DR5-DISC, beyond DR5 and caspase 8 upregulation. Many of the proteins involved in the DR-mediated apoptotic pathways are regulated by ubiquitination (53), and some of these proteins could play a crucial role in specific regulation of DR5 signaling. Binding of the DEAD-box RNA helicase DDX3 to a non-death domain region of DR5 was found to specifically mediate resistance to a DR5-targeting antibody (TRA-8) in the TRA-8-resistant MDA-MB-231R breast cancer cell line (54). Understanding how MG-132 in particular can overcome DR5-resistance may be of crucial importance since Johnsen *et al.* previously reported that IFN- γ failed to increase TRAIL-induced apoptosis in 3 out of 8 neuroblastoma cancer cell lines, despite caspase 8 upregulation and presence of all the proteins known to be necessary for DISC formation (40). It would be interesting to verify whether DR4 signalling is functional in the non-responding cells. Furthermore, in view of the large number of agents in clinical development targeting DR5 as opposed to DR4, a deeper knowledge on DR5-mediated signaling regulation is critical for a more rational design of therapies aiming specifically at DR5-mediated apoptosis.

In summary, we have demonstrated that cell sensitivity to rhTRAIL signaling via DR4 and DR5 mostly coincided with sensitivity to agonistic anti-DR4 and anti-DR5 antibodies, respectively. Apoptosis induced by DR4 stimuli in SW948-TR cells seemed limited by the lesser amounts of caspase 8 available as compared to the parent cell line.

Resistance to DR5-mediated apoptosis in SW948-TR cells most likely stemmed from a combination of at least two mechanisms; (1) low caspase 8 levels and (2) sub-optimal capacity to process the existing caspase 8 at the level of this receptor, which was also seen in the parent cell line. Only MG-132 was able to restore generation of active caspase 8 in SW948-TR upon DR5-stimulation. These results underscore the DR-specificity of drug combination and the presence of different resistance mechanisms at the level of DR4 and DR5.

Acknowledgements

This work was supported by grant 2000-2286 from the Dutch Cancer Society.

References

1. Kelley SK and Ashkenazi A. Targeting death receptors in cancer with Apo2L/TRAIL. *Curr Opin Pharmacol* 2004; 4: 333-339.
2. Oldenhuis CNAM, Stegehuis JH, Walenkamp AME, de Jong S and de Vries EGE. Targeting TRAIL death receptors. *Curr Opin Pharmacol* 2008; 8: 433-439.
3. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008; 8: 915-928.
4. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC and Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* 1999; 5: 157-163.
5. Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, Ross BD and Rehemtulla A. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proc Natl Acad Sci U S A* 2000; 97: 1754-1759.
6. Gliniak B and Le T. Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity in vivo is enhanced by the chemotherapeutic agent CPT-11. *Cancer Res* 1999; 59: 6153-6158.
7. Shankar S, Singh TR and Srivastava RK. Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer in vitro and in vivo: Intracellular mechanisms. *Prostate* 2004; 61: 35-49.
8. Singh TR, Shankar S, Chen X, Asim M and Srivastava RK. Synergistic interactions of chemotherapeutic drugs and tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand on apoptosis and on regression of breast carcinoma in vivo. *Cancer Res* 2003; 63: 5390-5400.
9. Buchsbaum DJ, Zhou T, Grizzle WE, Oliver PG, Hammond CJ, Zhang S, Carpenter M and LoBuglio AF. Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. *Clin Cancer Res* 2003; 9: 3731-3741.
10. Chuntharapai A, Dodge K, Grimmer K, Schroeder K, Marsters SA, Koeppen H, Ashkenazi A and Kim KJ. Isotype-dependent inhibition of tumor growth in vivo by monoclonal antibodies to death receptor 4. *J Immunol* 2001; 166: 4891-4898.
11. Ichikawa K, Liu W, Zhao L, Wang Z, Liu D, Ohtsuka T, Zhang H, Mountz JD, Koopman WJ, Kimberly RP and Zhou T. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat Med* 2001; 7: 954-960.
12. Ichikawa K, Liu W, Fleck M, Zhang H, Zhao L, Ohtsuka T, Wang Z, Liu D, Mountz JD, Ohtsuki M, Koopman WJ, Kimberly R and Zhou T. TRAIL-R2 (DR5) mediates apoptosis of synovial fibroblasts in rheumatoid arthritis. *J Immunol* 2003; 171: 1061-1069.
13. Kelley SK, Harris LA, Xie D, DeForge L, Totpal K, Bussiere J and Fox JA. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans:

- characterization of in vivo efficacy, pharmacokinetics, and safety. *J Pharmacol Exp Ther* 2001; 299: 31-38.
14. Plummer R, Attard G, Pacey S, Li L, Razak A, Perrett R, Barrett M, Judson I, Kaye S, Fox NL, Halpern W, Corey A, Calvert H and de BJ. Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers. *Clin Cancer Res* 2007; 13: 6187-6194.
 15. Tolcher AW, Mita M, Meropol NJ, von MM, Patnaik A, Padavic K, Hill M, Mays T, McCoy T, Fox NL, Halpern W, Corey A and Cohen RB. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *J Clin Oncol* 2007; 25: 1390-1395.
 16. Georgakis GV, Li Y, Humphreys R, Andreeff M, O'Brien S, Younes M, Carbone A, Albert V and Younes A. Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. *Br J Haematol* 2005; 130: 501-510.
 17. Kelley RF, Totpal K, Lindstrom SH, Mathieu M, Billeci K, DeForge L, Pai R, Hymowitz SG and Ashkenazi A. Receptor-selective mutants of apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling. *J Biol Chem* 2005; 280: 2205-2212.
 18. Zheng B, Georgakis GV, Li Y, Bharti A, McConkey D, Aggarwal BB and Younes A. Induction of cell cycle arrest and apoptosis by the proteasome inhibitor PS-341 in Hodgkin disease cell lines is independent of inhibitor of nuclear factor-kappaB mutations or activation of the CD30, CD40, and RANK receptors. *Clin Cancer Res* 2004; 10: 3207-3215.
 19. Kischkel FC, Lawrence DA, Tinel A, LeBlanc H, Virmani A and Schow P. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J Biol Chem* 2001; 276: 46639-46646.
 20. Merino D, Lalaoui N, Morizot A, Schneider P, Solary E and Micheau O. Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2. *Mol Cell Biol* 2006; 26: 7046-7055.
 21. Griffith TS, Rauch CT, Smolak PJ, Waugh JY, Boiani N, Lynch DH, Smith CA, Goodwin RG and Kubin MZ. Functional analysis of TRAIL receptors using monoclonal antibodies. *J Immunol* 1999; 162: 2597-2605.
 22. Pukac L, Kanakaraj P, Humphreys R, Alderson R, Bloom M, Sung C, Riccobene T, Johnson R, Fiscella M, Mahoney A, Carrell J, Boyd E, Yao XT, Zhang L, Zhong L, von KA, Shepard L, Vaughan T, Edwards B, Dobson C, Salcedo T and Albert V. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types in vitro and in vivo. *Br J Cancer* 2005; 92: 1430-1441.
 23. Thomas LR, Johnson RL, Reed JC and Thorburn A. The C-terminal tails of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas receptors have opposing functions in Fas-associated death domain (FADD) recruitment and can regulate agonist-specific mechanisms of receptor activation. *J Biol Chem* 2004; 279: 52479-52486.

24. He Q, Huang Y and Sheikh MS. Proteasome inhibitor MG132 upregulates death receptor 5 and cooperates with Apo2L/TRAIL to induce apoptosis in Bax-proficient and -deficient cells. *Oncogene* 2004; 23: 2554-2558.
25. Ruiz de Almodovar C, Lopez-Rivas A and Ruiz-Ruiz C. Interferon-gamma and TRAIL in human breast tumor cells. *Vitam Horm* 2004; 67: 291-318.
26. Yang X, Merchant MS, Romero ME, Tsokos M, Wexler LH, Kontny U, Mackall CL and Thiele CJ. Induction of caspase 8 by interferon gamma renders some neuroblastoma (NB) cells sensitive to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) but reveals that a lack of membrane TR1/TR2 also contributes to TRAIL resistance in NB. *Cancer Res* 2003; 63: 1122-1129.
27. Fulda S and Debatin KM. 5-Aza-2'-deoxycytidine and IFN-gamma cooperate to sensitize for TRAIL-induced apoptosis by upregulating caspase-8. *Oncogene* 2006; 25: 5125-5133.
28. Kabore AF, Sun J, Hu X, McCrea K, Johnston JB and Gibson SB. The TRAIL apoptotic pathway mediates proteasome inhibitor induced apoptosis in primary chronic lymphocytic leukemia cells. *Apoptosis* 2006; 11: 1175-1193.
29. Li W, Zhang X and Olumi AF. MG-132 sensitizes TRAIL-resistant prostate cancer cells by activating c-Fos/c-Jun heterodimers and repressing c-FLIP(L). *Cancer Res* 2007; 67: 2247-2255.
30. Merchant MS, Yang X, Melchionda F, Romero M, Klein R, Thiele CJ, Tsokos M, Kontny HU and Mackall CL. Interferon gamma enhances the effectiveness of tumor necrosis factor-related apoptosis-inducing ligand receptor agonists in a xenograft model of Ewing's sarcoma. *Cancer Res* 2004; 64: 8349-8356.
31. Varela N, Munoz-Pinedo C, Ruiz-Ruiz C, Robledo G, Pedroso M and Lopez-Rivas A. Interferon-gamma sensitizes human myeloid leukemia cells to death receptor-mediated apoptosis by a pleiotropic mechanism. *J Biol Chem* 2001; 276: 17779-17787.
32. Zhu H, Guo W, Zhang L, Wu S, Teraishi F, Davis JJ, Dong F and Fang B. Proteasome inhibitors-mediated TRAIL resensitization and Bik accumulation. *Cancer Biol Ther* 2005; 4: 781-786.
33. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z and Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999; 104: 155-162.
34. Leibovitz A, Stinson JC, McCombs WB, III, McCoy CE, Mazur KC and Mabry ND. Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* 1976; 36: 4562-4569.
35. van Geelen CMM, de Vries EGE, Le TKP, van Weeghel RP and de Jong S. Differential modulation of the TRAIL receptors and the CD95 receptor in colon carcinoma cell lines. *Br J Cancer* 2003; 89: 363-373.
36. Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P, Blenis J and Tschopp J. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* 2000; 2: 241-243.
37. Krammer PH, Arnold R and Lavrik IN. Life and death in peripheral T cells. *Nat Rev Immunol* 2007; 7: 532-542.

38. Hougardy BM, Maduro JH, van der Zee AG, de Groot DJ, van den Heuvel FA, de Vries EGE and de Jong S. Proteasome inhibitor MG132 sensitizes HPV-positive human cervical cancer cells to rhTRAIL-induced apoptosis. *Int J Cancer* 2006; 118: 1892-1900.
39. Yoshida T, Shiraishi T, Nakata S, Horinaka M, Wakada M, Mizutani Y, Miki T and Sakai T. Proteasome inhibitor MG132 induces death receptor 5 through CCAAT/enhancer-binding protein homologous protein. *Cancer Res* 2005; 65: 5662-5667.
40. Johnsen JI, Pettersen I, Ponthan F, Sveinbjornsson B, Flaegstad T and Kogner P. Synergistic induction of apoptosis in neuroblastoma cells using a combination of cytostatic drugs with interferon-gamma and TRAIL. *Int J Oncol* 2004; 25: 1849-1857.
41. Pennarun B, Meijer A, de Vries EGE, Kleibeuker JH, Kruyt FA and de Jong S. Playing the DISC: Turning on TRAIL death receptor-mediated apoptosis in cancer. *BBA-Rev Cancer* 2010; 1805: 123-140.
42. Leverkus M, Sprick MR, Wachter T, Denk A, Bocker EB, Walczak H and Neumann M. TRAIL-induced apoptosis and gene induction in HaCaT keratinocytes: differential contribution of TRAIL receptors 1 and 2. *J Invest Dermatol* 2003; 121: 149-155.
43. van der Sloot AM, Tur V, Szegezdi E, Mullally MM, Cool RH, Samali A, Serrano L and Quax WJ. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. *Proc Natl Acad Sci U S A* 2006; 103: 8634-8639.
44. Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lill JR and Ashkenazi A. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell* 2009; 137: 721-735.
45. Yang X, Merchant MS, Romero ME, Tsokos M, Wexler LH, Kontny U, Mackall CL and Thiele CJ. Induction of caspase 8 by interferon gamma renders some neuroblastoma (NB) cells sensitive to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) but reveals that a lack of membrane TR1/TR2 also contributes to TRAIL resistance in NB. *Cancer Res* 2003; 63: 1122-1129.
46. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002; 2: 420-430.
47. Geller J, Petak I, Szucs KS, Nagy K, Tillman DM and Houghton JA. Interferon-gamma-induced sensitization of colon carcinomas to ZD9331 targets caspases, downstream of Fas, independent of mitochondrial signaling and the inhibitor of apoptosis survivin. *Clin Cancer Res* 2003; 9: 6504-6515.
48. Langaas V, Shahzidi S, Johnsen JI, Smedsrod B and Sveinbjornsson B. Interferon-gamma modulates TRAIL-mediated apoptosis in human colon carcinoma cells. *Anticancer Res* 2001; 21: 3733-3738.
49. Lavrik IN, Golks A, Riess D, Bentele M, Eils R and Krammer PH. Analysis of CD95 threshold signaling: triggering of CD95 (FAS/APO-1) at low concentrations primarily results in survival signaling. *J Biol Chem* 2007; 282: 13664-13671.
50. Smith MR, Jin F and Joshi I. Bortezomib sensitizes non-Hodgkin's lymphoma cells to apoptosis induced by antibodies to tumor necrosis factor related apoptosis-inducing ligand (TRAIL) receptors TRAIL-R1 and TRAIL-R2. *Clin Cancer Res* 2007; 13: 5528s-5534s.

51. Koschny R, Sykora J, Walczak H, Ganten T, Haas T, Sprick M, Holland H, Ahnert P, Krupp W, Meixensberger J+ and Bauer M. Bortezomib-Mediated Up-Regulation of TRAIL-R1 and TRAIL-R2 Is Not Necessary for but Contributes to Sensitization of Primary Human Glioma Cells to TRAIL. *Clinical Cancer Res* 2007; 13: 6541-6542.
52. Liu X, Yue P, Chen S, Hu L, Lonial S, Khuri FR and Sun SY. The proteasome inhibitor PS-341 (bortezomib) up-regulates DR5 expression leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. *Cancer Res* 2007; 67: 4981-4988.
53. Zhang HG, Wang J, Yang X, Hsu HC and Mountz JD. Regulation of apoptosis proteins in cancer cells by ubiquitin. *Oncogene* 2004; 23: 2009-2015.
54. Li Y, Wang H, Wang Z, Makhija S, Buchsbaum D, LoBuglio A, Kimberly R and Zhou T. Inducible Resistance of Tumor Cells to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor 2-Mediated Apoptosis by Generation of a Blockade at the Death Domain Function. *Cancer Res* 2006; 66: 8520-8528.

Inhibition of tyrosine kinase-dependent PI3K activation sensitizes colon cancer cells specifically to DR5-mediated apoptosis but not to rhTRAIL

Bodvaël Pennarun¹

Jan H. Kleibeuker²

Tjitske Oenema^{1,3}

Janet H. Stegehuis¹

Elisabeth G.E. de Vries¹

Steven de Jong¹

Departments of ¹Medical Oncology, ²Gastroenterology, and ³Molecular Pharmacology,

^{1,2}University Medical Center Groningen, 9713 GZ Groningen,

³University of Groningen, the Netherlands

Submitted for publication

Abstract

Background: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) initiates apoptosis in tumor cells upon binding to its cognate agonistic receptors, death receptors 4 and 5 (DR4 and DR5). The activity of the insulin-like growth factor 1 (IGF-1) survival pathway is often increased in cancer, influencing both cell proliferation and apoptosis. We hypothesized that inhibiting the IGF-1 receptor (IGF-1R) using NVP-AEW541, a small molecular weight tyrosine kinase inhibitor of the IGF-1R, could increase death receptor (DR)-mediated apoptosis in colon cancer cells. Methods: The analyses were performed by caspase assay, flow cytometry, Western blotting, immunoprecipitation and fluorescent microscopy. Results: Preincubation with NVP-AEW541 surprisingly decreased apoptosis induced by recombinant human TRAIL (rhTRAIL) or an anti-DR4 antibody while sensitivity to an anti-DR5 antibody was increased. NVP-AEW541 could inhibit IGF-1-induced activation of the phosphatidylinositol 3-kinase (PI3K) pathway. The effects of the PI3K inhibitor LY294002 on TRAIL-induced apoptosis were similar to those of NVP-AEW541, further supporting a role for IGF-1R-mediated activation of PI3K. We show that PI3K inhibition enhances DR5-mediated caspase 8 processing but also lowers DR4 membrane expression and DR4-mediated caspase 8 processing. Inhibition of PI3K reduced rhTRAIL sensitivity independently of the cell line preference for either DR4 or DR5-mediated apoptosis signaling. Conclusions: Our study indicates that individual effects on DR4 and DR5 apoptosis signaling should be taken into consideration when combining DR-ligands with PI3K inhibition.

Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in western countries. Despite improvements in the area of prevention and treatment, mortality remains high because of the frequent presence of distant metastases at the time of diagnosis. At that stage, treatment results in a five year survival of only 10% to 30% (1). New therapies are greatly needed, and novel agents like those targeting the TRAIL apoptotic pathway are currently under investigation (2).

The TRAIL apoptotic pathway is initiated by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a type II transmembrane protein of the tumor necrosis factor superfamily. TRAIL binds to cell surface homo- and/or heterotrimers of death receptors 4 and 5 (DR4 and DR5), thus triggering the “caspase cascade” (3). The extracellular domain of TRAIL can be processed proteolytically, leading to the release of a soluble trimeric form of TRAIL with similar proapoptotic properties (4,5). The potential of the TRAIL apoptotic pathway as a target to treat cancer was first established when soluble recombinant human TRAIL (rhTRAIL) was found to selectively induce apoptosis in tumor cells without serious toxicity to normal tissues (6,7). Several other agents that target the two death receptors have been engineered since the discovery of the TRAIL apoptotic pathway. So far, this pathway can be activated by rhTRAIL, by mutant forms of TRAIL which bind preferentially to either DR4 or DR5 homotrimers (8-10), and by agonistic monoclonal antibodies also designed to bind specifically DR4 or DR5 (11-14). The potential of the TRAIL receptors as therapeutic targets is underscored by the fact that their expression and sensitivity have both been shown to increase during colorectal tumor progression (15,16). Although the sensitivity of tumor cells to TRAIL-induced apoptosis is heterogeneous as a result of intrinsic or acquired resistance, combination therapies to overcome this phenomenon are already under investigation (for review see (17,18)).

Numerous growth factor receptor pathways are altered in colorectal cancer, influencing gene expression, cell proliferation and apoptosis (19). Some of these pathways constitute interesting targets for drugs that could be combined with rhTRAIL (20-22). The insulin-like growth factor (IGF) signaling pathway is involved in the growth and development of many tissues, and plays a crucial role in the normal functioning of the organism (23). IGF signaling cascades begin at the cell surface with IGF ligands (IGF-

1 and -2) binding to several transmembrane receptors, namely IGF-1R, IGF-2R and the insulin receptor (IR). Downstream from these receptors, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways are stimulated (23). Diverse cellular responses such as gene expression, cell proliferation and apoptosis suppression are induced, thus promoting cell survival. Importantly, the IGF system is frequently deregulated in colorectal cancers (23,24), more than 90% of which express IGF-1R while normal colonic mucosa does not show substantial expression levels (25).

The downstream PI3K/Akt receives signals not only from the IGF-1R but also from human epidermal growth factor receptor (HER) family receptors such as HER-2 and epidermal growth factor receptor (EGF-R) (26,27), which can also promote carcinogenesis when deregulated. Furthermore, alterations in the PI3K/Akt signaling itself have been found in many forms of cancer and can originate at multiple levels of the cascade (28,29). For these reasons, the PI3K/Akt pathway holds promise as a target for cancer treatment. Clinical trials are being carried out with IGF-1R blocking antibodies and inhibitors targeting PI3K are currently being developed (30,31). In this context, we investigated whether inhibiting the IGF-1R survival pathway, and more specifically its downstream PI3K/Akt pathway, could functionally increase rhTRAIL-induced apoptosis.

We tested the effects of the IGF-1R inhibitor NVP-AEW541 (32) on TRAIL-sensitivity in SW948, a colon cancer cell line and in SW948-TR, the TRAIL-resistant sub-line. Furthermore, we investigated whether sensitivity to DR4- or DR5-specific monoclonal antibodies was differentially affected by NVP-AEW541. We analyzed the effects of LY294002, an inhibitor of the downstream PI3K/Akt pathway, on the sensitivity to these various activators of TRAIL-mediated cell death. Finally, we validated these findings in another colon carcinoma cell line, Colo205.

Materials and methods

Reagents

rhTRAIL was produced non-commercially following a protocol described previously (6). The agonistic anti-DR4 (HGS-ETR1) and anti-DR5 (HGS-TR2J) antibodies were a gift from Human Genome Science (HGS, Rockville, MD, USA). The TRAIL-receptor antibodies used for flow cytometry experiments were obtained from Immunex

corporation (Seattle, WA, USA). NVP-AEW541 was kindly provided by Novartis Pharma AG, Basel, Switzerland. LY294002 was purchased from Cell Signaling Technology, Leiden, the Netherlands. The ZB ActiveFluor Caspase 3/7 kits were obtained from Zebra Bioscience, Enschede, the Netherlands. 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT)-solution was purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands)

Cell lines

The TRAIL-sensitive colon carcinoma cell line SW948 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as described previously (33). For a detailed description of the generation of the TRAIL-resistant sub cell line SW948-TR, see supplementary materials and methods.

Colo205 colon carcinoma and MCF7 breast carcinoma cell lines were purchased from the ATCC and grown in RPMI containing 10% FCS at 37 °C in a humidified atmosphere with 5% CO₂.

Cytotoxicity assay

A microculture tetrazolium (MTT) assay was used to determine cytotoxicity. SW948 and SW948-TR cells were incubated in a total volume of 200 µl. Treatment consisted of continuous incubation with various concentrations of NVP-AEW541 for 96h. Assays were performed as described before (33).

Flow cytometry

Cells were stained for TRAIL-receptor membrane expression as described previously (33) and analyzed with a Epics Elite flow cytometer (Coulter Electronics, Hialeah, FL, USA). Membrane expression was measured as the increase in mean fluorescence intensity of the whole analyzed cell population.

To determine surface expression of IGF-1R, a similar protocol was used. Cells were stained for 45 min on ice with phycoerythrin(PE)-conjugated anti-human IGF-1R α or PE-conjugated mouse IgG1 as an isotype control (BD Pharmingen, Alphen aan den Rijn, the Netherlands). After two washes a minimum of 5,000 cells was analyzed by flow cytometry.

Caspase 3/7 activity assay

Caspase 3/7 activity was assayed using the caspase-specific fluorescence peptide substrates DEVD-MCA according to the manufacturer's instructions (Zebra Bioscience BV, Groningen, the Netherlands). A Bradford assay was used to normalize protein concentrations between samples. Fluorescence from free 7-amino-4-trifluoromethyl coumarin was monitored in a FL600 Fluorimeter Bio-tek plate reader (Beun de Ronde, Abcoude, the Netherlands) using 340 nm excitation and 460 nm emission filters.

SDS-polyacrylamide gel electrophoresis and Western blotting

Sample preparation and Western blot analysis were performed as described previously (33). Proteins were detected with the following antibodies: mouse-anti-FADD from Transduction Laboratories (Lexington, KY, USA), mouse anti-caspase 8 from Cell Signaling Technology (Leusden, the Netherlands) and mouse anti-actin from ICN Biomedicals (Zoetermeer, the Netherlands). Mouse-anti-FLIP NF6 was kindly provided by Dr. M. Peter (University of Chicago, IL, USA). The secondary antibodies were labeled with horseradish peroxidase (HRP) (DAKO, Glostrup, Denmark), and chemiluminescence was detected using the BM-chemiluminescence kit or with the Lumi-Light Plus Western blotting kit (Roche Diagnostics, Mannheim, Germany). A slightly modified lysis protocol was used to investigate Akt, MAPK^{44/42}, phospho-MAPK^{44/42} and phospho-Akt (ser473) protein levels (see supplementary material and methods).

DR5-DISC isolation

Briefly, 50.10⁶ cells per condition were grown in medium harvested and resuspended in fresh medium. Cells were stimulated with 5 µg/ml HGS-TR2J in a final volume of 2 ml. The antibodies were added after cell lysis for the control treatment. Cell suspensions were incubated for 15 min at 37 °C, and the reaction was stopped by the addition of 10 ml ice-cold phosphate buffered saline (PBS). The cells were immediately washed with ice-cold PBS and lysed in 1 ml lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) with complete protease inhibitors (Roche Diagnostics, Almere, the Netherlands) for 30 min on ice. After centrifugation (12,000 × g) at 4 °C for 10 min, the lysates were pre-cleared with 20 µl Sepharose-6B (Pharmacia, Uppsala, Sweden) for 2 h at 4 °C and immunoprecipitated

with 50 µl protein-G agarose beads (Roche Diagnostics, Mannheim, Germany) for 3 at 4 °C. Beads were washed two times with 1 ml lysis buffer and one time with PBS before re-suspension in standard Western blot sample buffer and boiling for 5 min. Immunoprecipitated proteins were separated with SDS-PAGE. Western blot analysis for FADD and c-FLIP was performed as described above. DR5 was detected using rabbit anti-DR5 from ProSci Inc. (Poway, CA, USA). Caspase 8 was detected with rabbit anti-caspase 8 (Abcam plc, Cambridge, UK). Anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were used for signal detection (DAKO, Glostrup, Denmark).

Propidium iodide staining

Staining was performed according to Nicoletti's protocol (34). Sub-G₁ DNA content was analyzed using a FACSCalibur flow cytometer (BD Biosciences, Brussels, Belgium).

Fluorescent microscopy

SW948 cells were seeded in 6-well plate on poly-L-lysine coated glass cover slides (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). Cells pretreated or not with LY294002 for 16 hours were incubated for 45 min on ice with anti-DR5 antibody (eBiosciences, via ITK diagnostics BV, Uithoorn, the Netherlands), diluted 1:100 in ice-cold PBS. After two washing steps with ice-cold PBS, the cells were incubated with the secondary antibody Alexa Fluor 488-goat anti-mouse IgG1 (Invitrogen, Molecular probes, Breda, the Netherlands), diluted 1:200 in ice-cold PBS for 1 hour on ice. Next, cells were either left on ice or stimulated with 200 nM HGS-TR2J diluted in medium for 30 min at 37 °C. Cells were then fixated with ice-cold methanol-acetone (1:1) for 10 min at -20°C. After three washing steps with ice-cold PBS, the cells were stained with Hoechst 33258 (Invitrogen, Molecular Probes, Leiden, the Netherlands) for 5 min on ice, diluted 1:10 000 in ice-cold PBS to visualize the nuclei. After mounting the slides in Vectashield Mounting medium H-1000 (Vector, via Brunschwig Chemie, Amsterdam, the Netherlands), analysis was performed using a Leica fluorescence microscope DM-RXA.

Statistical analysis

Data are represented as the mean \pm SE. In all cases, statistical analyses were done using 2-tailed student's *t* test. *P*-values < 0.05 were considered significant and are indicated with an asterisk.

Results

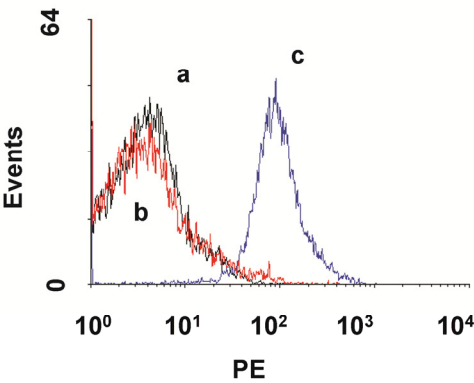
IGF-1R inhibition using NVP-AEW541 modulates sensitivity to rhTRAIL and DR-agonistic antibodies

We evaluated the role of IGF-1R expression and its inhibition on TRAIL sensitivity using the isogenic SW948/SW948-TR model. Membrane expression of IGF-1R was similar in the SW948 cell line and its TRAIL resistant sub-line SW948-TR (Fig. 1A-B). The surface levels were not as elevated as those seen in the MCF7 cells, known to express high levels of IGF-1R (Fig. 1B). We then incubated the cells with various concentrations of the IGF-1R inhibitor NVP-AEW541. Both cell lines showed nanomolar sensitivity to the compound, with a mean GI₄₀ (concentration inhibiting cell growth by 40%) below 1 μ M in a survival assay (Fig. 1C).

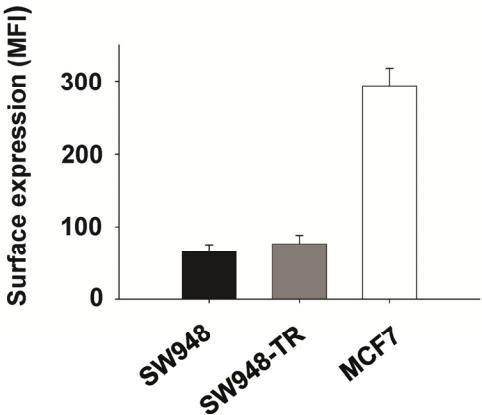
NVP-AEW541 was combined at a concentration corresponding to the GI₆₀ with rhTRAIL or the DR-agonistic antibodies to stimulate apoptosis. Cleavage of poly-ADP ribose phosphate (PARP) was used to assess apoptosis in SW948 (Fig. 1D). The untreated cells and the cells incubated with NVP-AEW541 alone only exhibited the full form of PARP, indicating that NVP-AEW541 does not induce apoptosis at the concentration used. Upon treatment with rhTRAIL PARP was fully cleaved into its 89 kDa fragment, reflecting high levels of apoptosis. To study DR4 and DR5-mediated

Figure 1: SW948 and SW948-TR cells express surface IGF-1R, and inhibition of this receptor modifies sensitivity to death receptor agonists. **A:** Representative example of IGF-1R expression in SW948. Positive receptor expression was detected as an increased fluorescence intensity of the whole cell population and resulted in a peak-shift to the right (a: control; b: IgG1 and c: IGF-1R). **B:** Surface expression of IGF-1R in SW948, SW948-TR and MCF7 cells (positive control for IGF-1R expression), as determined by flow cytometry. IGF-1R expression is given as Mean Fluorescent Intensity (MFI), normalized to 100. Values are mean \pm SE of at least 3 independent experiments. **C:** Survival (%) of SW948 and SW948-TR cells after continuous incubation with NVP-AEW541 for 96 h, as measured by cytotoxicity assays. Values are mean \pm SD of at least three independent experiments. **D:** Western blot analysis of PARP cleavage in SW948. The cells were pre-incubated for 15-18 hours with 5 μ M NVP-AEW541, then either left untreated or exposed for 3 additional hours with rhTRAIL (0.1 μ g/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) before harvest.

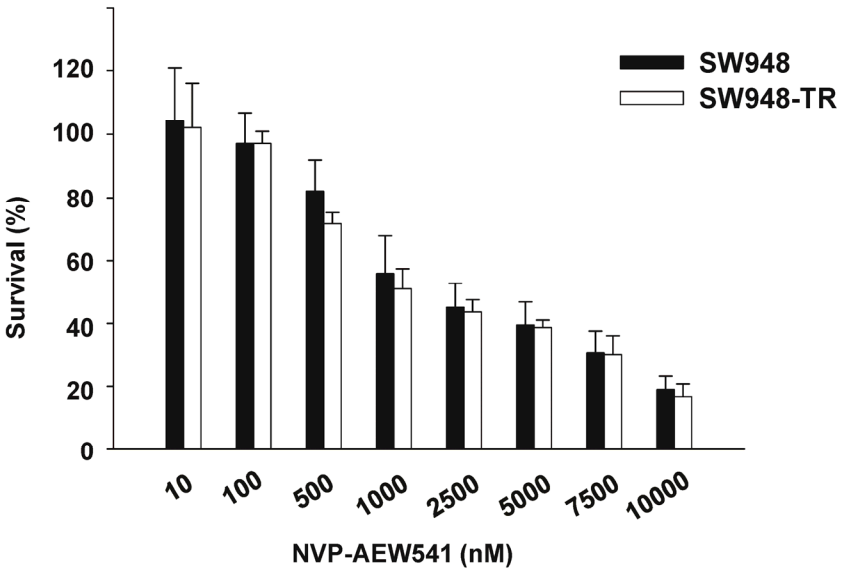
A



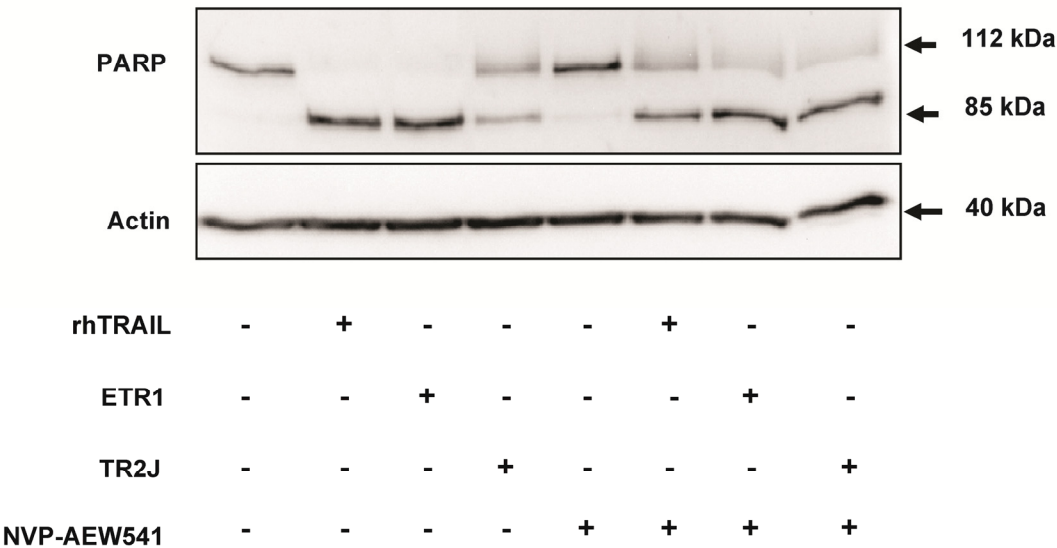
B



C



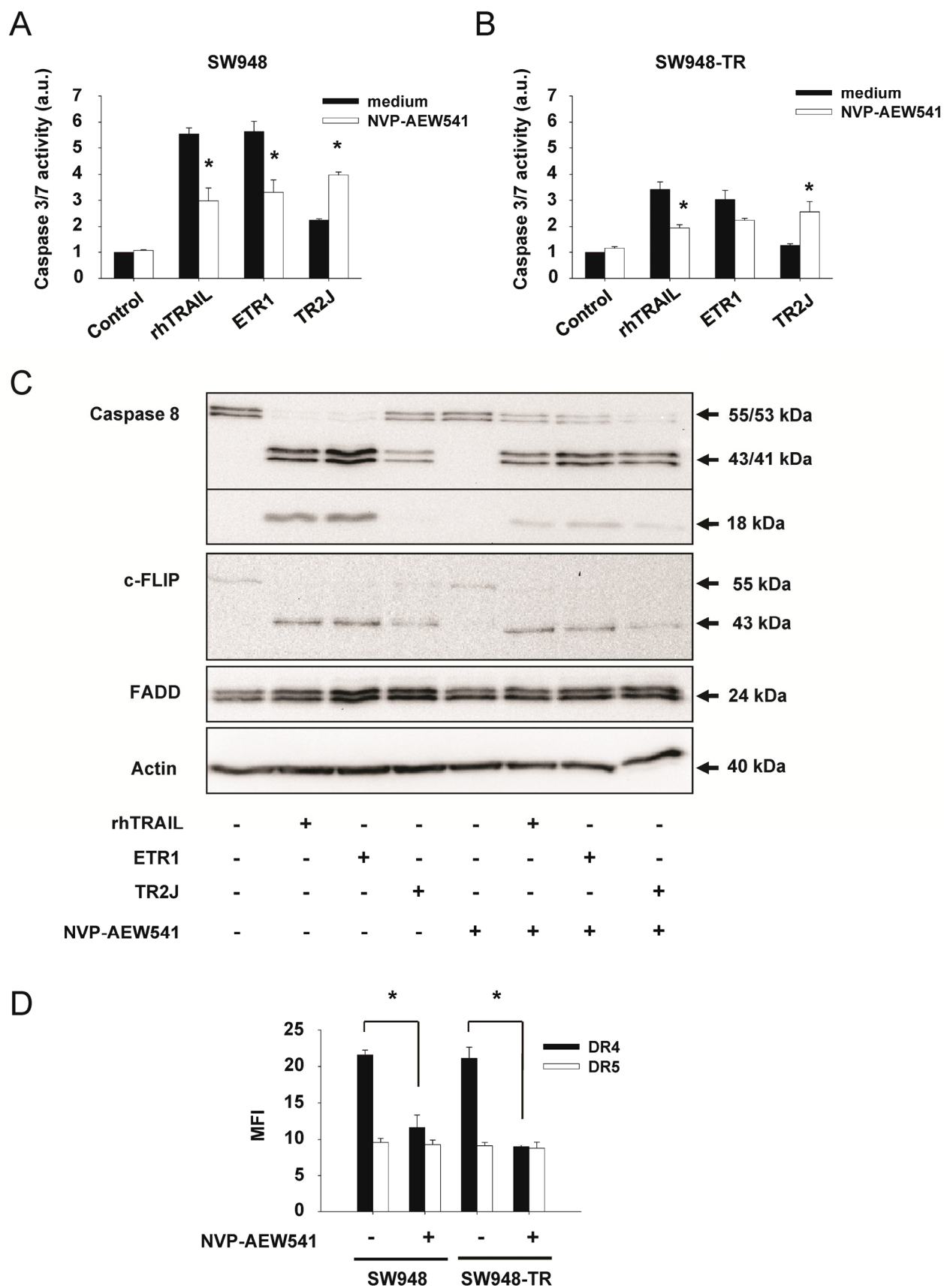
D



apoptosis individually, cells were stimulated with agonistic DR4 (HGS-ETR1) or DR5 antibody (HGS-TR2J). Similarly to rhTRAIL, HGS-ETR1 was a very effective inducer of PARP cleavage. HGS-TR2J also induced PARP cleavage, but only partially, indicating that DR5 was less potent than DR4 in transducing the apoptotic signal. When NVP-AEW541 was combined with rhTRAIL or HGS-ETR1, the cleavage of PARP was unexpectedly lower compared to rhTRAIL or HGS-ETR1 alone, which suggested that NVP-AEW541 decreased the sensitivity to these ligands. In contrast, the amount of cleaved PARP was higher when HGS-TR2J was combined with NVP-AEW541 in comparison to HGS-TR2J alone, signifying an increase in DR5-mediated apoptosis. Next, we performed caspase 3/7 enzyme activity assay as an additional method to assess apoptosis in SW948, and also in the TRAIL-resistant SW948-TR cells. Incubation with NVP-AEW541 alone had no effect on caspase 3/7 activation in either cell line (Fig. 2A-B). Upon inhibition of IGF-1R using NVP-AEW541, the sensitivity to rhTRAIL decreased by $46.2\% \pm 11.5$ (mean \pm SE) in SW948. Similar findings were done in SW948-TR cells, which became even more resistant to rhTRAIL ($42.6\% \pm 2$ decrease in caspase 3/7 activity after rhTRAIL treatment). IGF-1R inhibition effectively decreased DR4-mediated caspase 3/7 activity by $39.8\% \pm 10.8$ in SW948 cells, while SW948-TR cells followed a similar trend (19.3% decrease ± 15.2). In contrast, both cell lines exhibited an increase in DR5-mediated caspase 3/7 activity (an increase of $78.2\% \pm 5.9$ in SW948 and of $92.7\% \pm 26.7$ in SW948-TR).

Although we do not show a formal concentration-dependent analysis here, the effects of NVP-AEW541 on DR-mediated apoptosis proved to be concentration dependent. Concentrations below the GI_{60} showed similar, although less pronounced

Figure 2: IGF-1R inhibition modulates DR-mediated apoptosis and downregulates surface DR4 expression. Modulation of rhTRAIL or agonistic antibody induced apoptosis by NVP-AEW541 in SW948 (A) and SW948-TR (B), as assessed by caspase 3/7 activity assay. Cells were pre-incubated for 15-18 hours with 5 μ M NVP-AEW541, then either left untreated or exposed to rhTRAIL (0.1 μ g/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) for 3 additional hours before harvest. Caspase 3/7 activity is expressed in arbitrary units (a.u.), as the ratio of caspase 3/7 activity in treated cells to untreated cells. Values are mean \pm SE of at least 3 independent experiments. C: Western-blot analysis of the expression levels of several proteins located downstream from the TRAIL receptors, after IGF-1R inhibition and treatment with rhTRAIL or agonistic DR antibody, in SW948. The cells were pre-incubated for 15-18 hours with 5 μ M NVP-AEW541, then either left untreated or exposed for 3 additional hours with rhTRAIL (0.1 μ g/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) before harvest. D: Surface expression of TRAIL receptors in SW948 and SW948-TR, in control cells and after 15-18 hours exposure to 5 μ M NVP-AEW541.



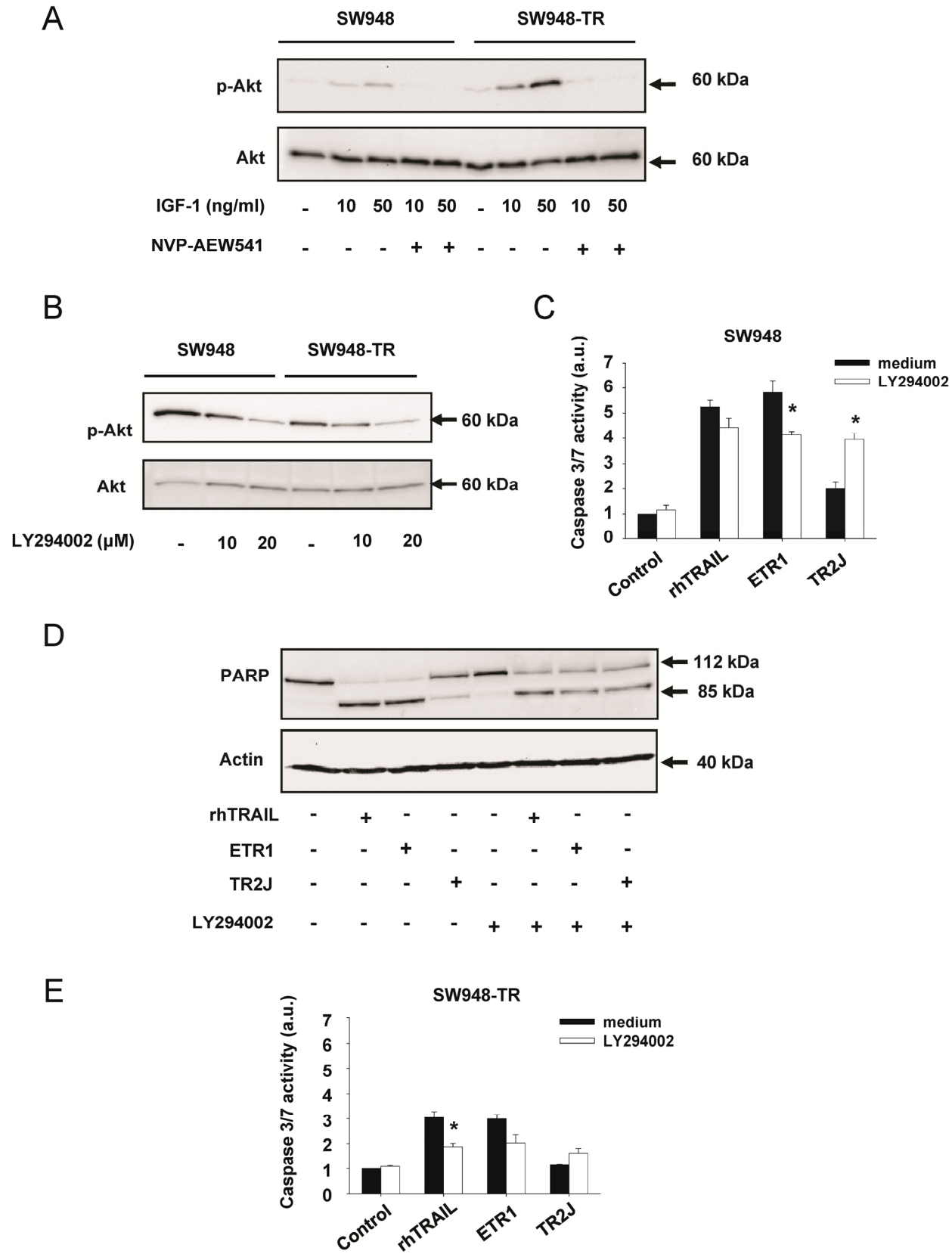


Figure 3: PI3K mediates the effects of IGF-1R inhibition on DR-mediated apoptosis. **A:** Western-blot analysis showing inhibition of IGF-1 induced Akt phosphorylation by NVP-AEW541 in SW948 and SW948-TR. Cells were pretreated for 1 hour with 5 μ M NVP-AEW541 before treatment with increasing concentrations of rhIGF-1 and subsequent harvesting. **B:** Western-blot analysis showing inhibition of Akt phosphorylation by LY294002 in SW948 and SW948-TR. Cells grown in medium with FCS were treated or not for 1 hour with increasing concentrations of LY294002 before harvest. **C:** Modulation of rhTRAIL or agonistic antibody induced apoptosis by PI3K inhibition in SW948, as assessed by caspase 3/7 activity assay in SW948. Cells were pre-incubated for 15-18 hours with 20 μ M LY294002, then either left untreated or exposed to rhTRAIL (0.1 μ g/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) for 3 additional hours before harvest. **C:** Caspase 3/7 activity assay in SW948 cells. Cells were pre-incubated for 15-18 hours with 20 μ M LY294002, then either left untreated or exposed to rhTRAIL (0.1 μ g/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) for 3 additional hours before harvest. **D:** Western blot analysis of PARP cleavage in SW948. The cells were pre-incubated for 15-18 hours with 20 μ M LY294002, then either left untreated or exposed for 3 additional hours with rhTRAIL (0.1 μ g/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) before harvest. **E:** Caspase 3/7 activity assay in SW948-TR. Cells were treated as described in (C).

effects, and higher concentrations of NVP-AEW541 (up to 10 μ M) had even more dramatic effects on DR-mediated apoptosis, in absence of additional cell death as a single agent (data not shown).

IGF-1R inhibition can modulate processing and expression of DISC components

The effects of NVP-AEW541 on the protein levels of the components of the death-inducing signaling complex (DISC) were analyzed by Western blotting. NVP-AEW541 did not decrease the basal expression of procaspase 8, nor did it affect the expression of fas-associated death domain (FADD) or cellular Flice inhibitory protein (c-FLIP), in SW948 (Fig. 2C). Following treatment with rhTRAIL, procaspase 8 was cleaved into its intermediate (p43/41) and ultimately active forms (p18). However, after pretreatment with NVP-AEW541, rhTRAIL failed to cause a complete cleavage of procaspase 8. As expected, c-FLIP was cleaved after treatment with rhTRAIL. NVP-AEW541 pretreatment did not induce any noticeable change in the processing of c-FLIP induced by rhTRAIL. After pretreatment with NVP-AEW541, HGS-ETR1 induced the same pattern of caspase 8 and c-FLIP content exhibited after the combination of rhTRAIL with NVP-AEW541. In contrast, DR5 targeting using HGS-TR2J alone failed to fully cleave procaspase 8 and c-FLIP. NVP-AEW541 pretreatment enhanced the responsiveness to HGS-TR2J, triggering almost full cleavage of both proteins. Such changes in the apoptotic response could be due to modulation of the surface expression of DR4 and DR5. We assessed the effects of NVP-AEW541 on cell membrane DR4 and DR5

expression in both SW948 and SW948-TR (Fig. 2D). Incubating the cells with NVP-AEW541 indeed resulted in a downregulation of membrane DR4. On the other hand, no changes were observed in DR5 membrane level.

IGF-1R exerts its effects on TRAIL sensitivity via the PI3K pathway

Because we expected NVP-AEW541 to influence DR-mediated apoptosis by inhibiting pathways downstream of IGF-1R, we tested whether IGF-1 could induce Akt phosphorylation, and whether NVP-AEW541 was able to inhibit this phosphorylation. Akt is one of the key proteins phosphorylated by PI3K downstream of IGF-1R (23). Cells were serum-starved for 24 hours, pretreated or not with NVP-AEW541 for one hour at a concentration neighboring the GI_{20} of both cell lines, and finally either left untreated or treated for 15 minutes with recombinant human (rh)IGF-1. Concentrations of rhIGF-1 (10-50 ng/mL) within the physiological range induced phosphorylation of Akt in both cell lines (Fig. 3A). Pretreatment with NVP-AEW541 dramatically reduced IGF-1 induced phosphorylation of Akt in both cell lines. Total Akt levels were similar in the two cell lines and were not affected by NVP-AEW541 or rhIGF-1. Phospho-MAPK^{44/42} (pMAPK^{44/42}) levels were studied as this protein can also be activated by phosphorylation in response to IGF-1. The levels of MAPK^{44/42} and pMAPK^{44/42} were similar with and without NVP-AEW541 treatment, suggesting that the activity of this pathway might be independent of IGF-1 in these cells (data not shown). We then decided to investigate whether a specific PI3K inhibitor could also inhibit Akt phosphorylation in SW948 and SW948-TR cells. As seen in Fig. 3B, upon treatment with 10-20 μ M LY294002 for 1 hour, Akt phosphorylation in both cell lines was inhibited in a concentration-dependent manner. LY294002 had no effects on total Akt expression levels.

Next, LY294002 was combined with rhTRAIL or death receptor-specific antibodies. Treatment of SW948 cells with LY294002 alone did not affect apoptosis, as measured by caspase 3/7 activity (Fig. 3C). HGS-ETR1-induced caspase 3/7 activity was reduced by $27.6\% \pm 8.2$. Although there was only a statistical trend, rhTRAIL-induced caspase 3/7 activity decreased when the cells were pretreated with LY294002 (by $14.7\% \pm 9.8$). In contrast, sensitivity through DR5 was clearly increased by $107.8\% \pm 38.5$. Analysis of PARP cleavage confirmed the changes in susceptibility to these agents in SW948 (Fig. 3D).

In SW948-TR (Fig. 3E), LY294002 similarly decreased sensitivity to rhTRAIL (by $38.3 \% \pm 0.6$), as assessed by caspase 3/7 activity measurement. Although these differences were not statistically significant, pretreatment with LY294002 lowered sensitivity to HGS-ETR1 (by $32.3 \% \pm 10.3$) but increased HGS-TR2J sensitivity ($41.2\% \pm 14.7$ increase).

PI3K inhibition modulates caspase 8 and c-FLIP cleavage

We examined whether LY294002 induced similar effects to NVP-AEW541 at the level of the DISC proteins. The results for caspase 8 were similar to those previously seen using NVP-AEW541, albeit the inhibition of procaspase 8 processing to the intermediate and active forms upon rhTRAIL and HGS-ETR1 seemed less strong (Fig. 4A). Basal c-FLIP and FADD levels were not affected by LY294002. The changes in c-FLIP cleavage after LY294002 exposure followed the pattern seen after NVP-AEW541 treatment, although the loss of c-FLIP through cleavage upon HGS-ETR1 or HGS-TR2J treatment after LY294002 was slightly higher.

Next, we looked whether inhibiting PI3K would modulate death receptor expression at the cell surface, as seen with NVP-AEW541. As expected, LY294002 downregulated DR4 to the level of DR5 in SW948 and SW948-TR, providing an explanation for the reduction in rhTRAIL sensitivity (Fig. 4B).

Localization of the death receptors is known to be modulated by certain drugs which cause them to aggregate and thereby increase their sensitivity to death inducing ligands (35). This prompted us to examine whether the distribution of DR5 at the cell surface was affected by PI3K inhibition. Fluorescent microscopy imaging of membrane DR5 in SW948 cells did not show any change in DR5 distribution at the cell surface following treatment with $20 \mu\text{M}$ LY294002 (Fig. 4C). Because differences in DR5 aggregation following LY294002 treatment might only occur after stimulation of the receptor, we stimulated DR5 for 30 minutes using HGS-TR2J. As seen in Fig. 4C, DR5 aggregated following stimulation with HGS-TR2J, but the staining pattern was similar between LY294002-treated and untreated cells.

Since PI3K inhibition increased caspase 8 activation, suggesting modifications in DR5-DISC formation, we performed a co-immunoprecipitation of this complex using HGS-TR2J, with or without pre-incubation with LY294002. To our surprise, DISC formation was comparable for both stimuli (Fig. 4D). HGS-TR2J could recruit DR5,

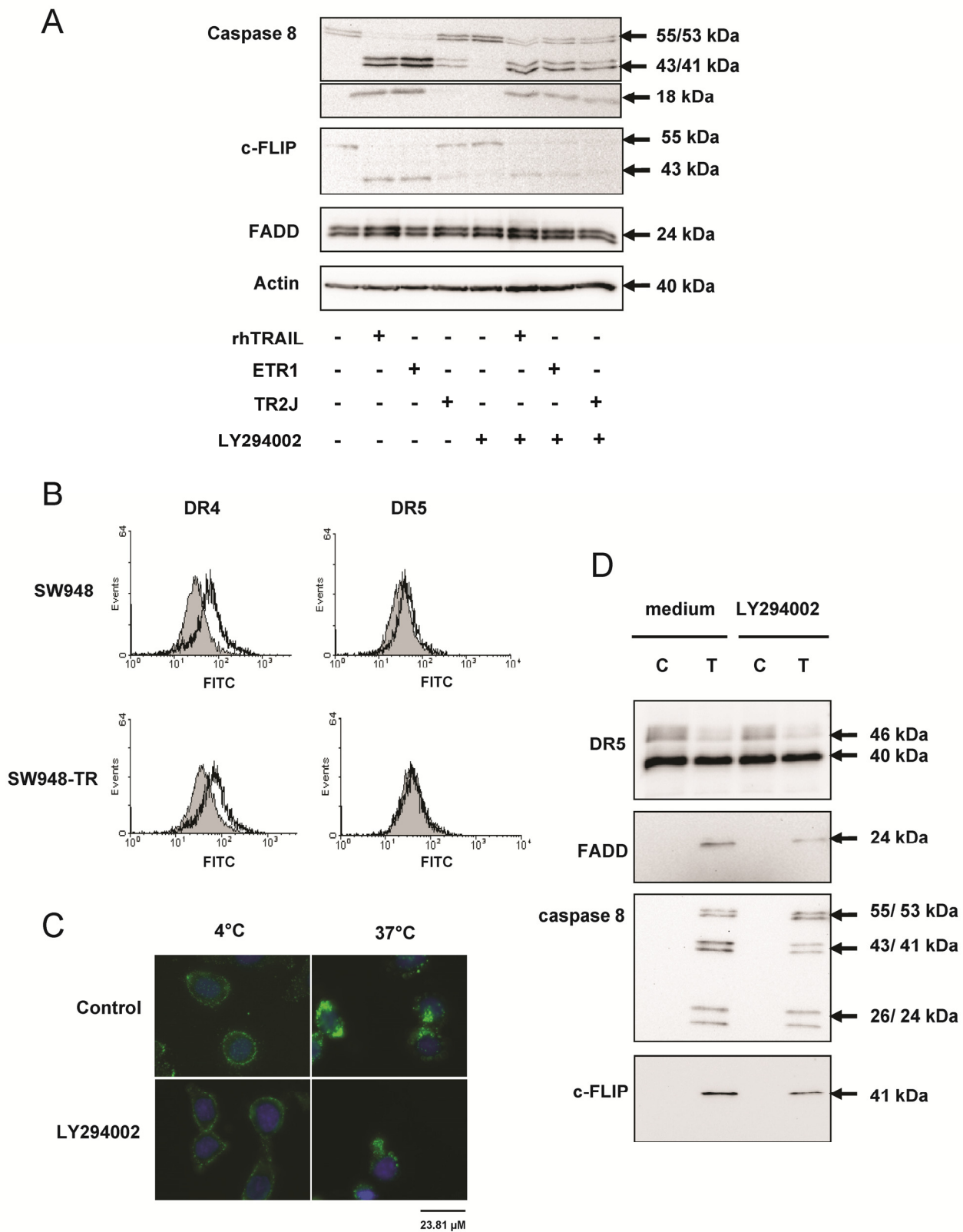


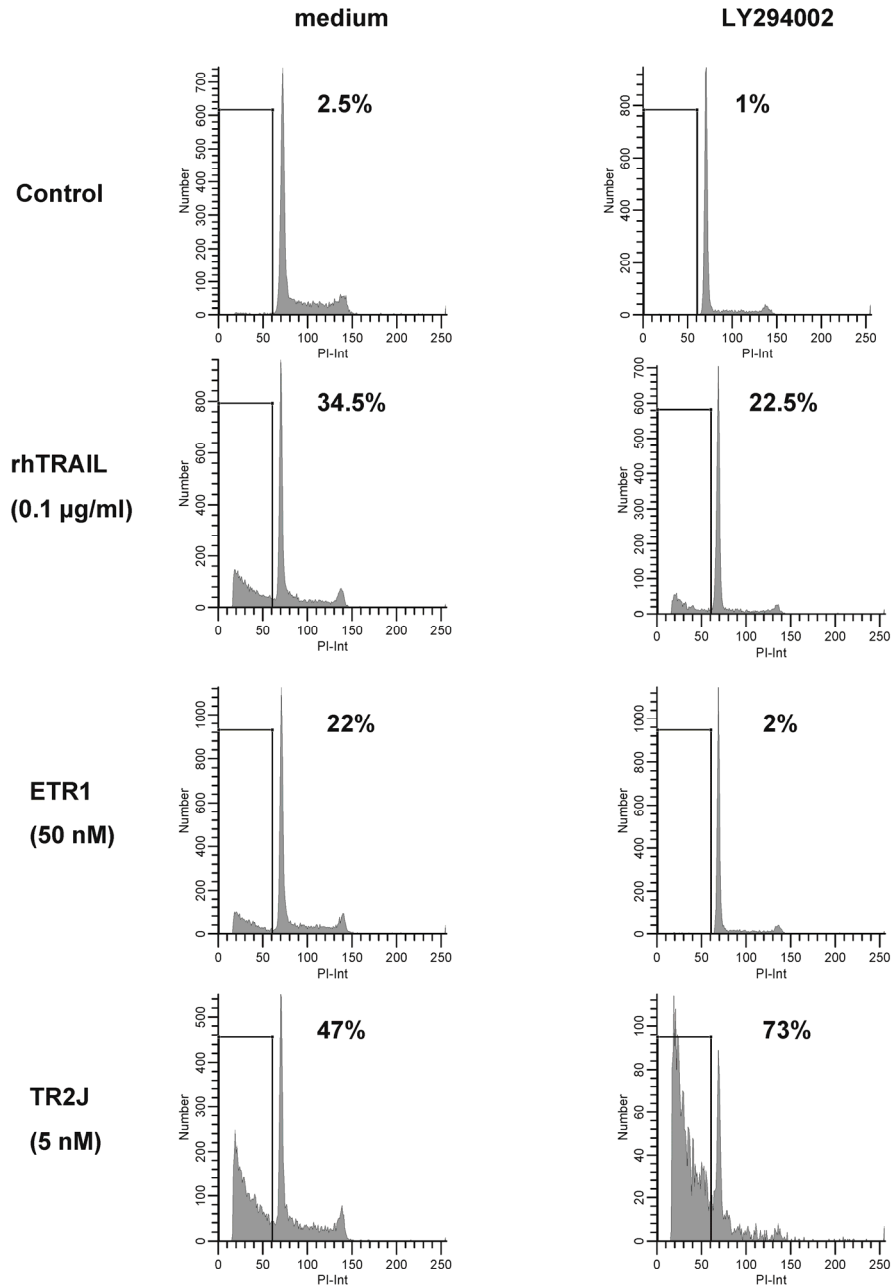
Figure 4: PI3K inhibition modulates DISC proteins cleavage and surface DR4 levels, but not cell membrane localization of DR5 or initial DR5-DISC formation. **A:** Expression levels of several proteins located downstream of the TRAIL receptors were determined by western-blotting in SW948. The cells were pre-incubated for 15-18 hours with 20 μ M LY294002, then either left untreated or exposed for 3 additional hours with rhTRAIL (0.1 μ g/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) before harvest. **B:** Surface expression of TRAIL receptors in SW948 and SW948-TR, in control cells and after 15-18 hours exposure to 20 μ M LY294002. Receptor expression was detected as the average antigenic density of the whole cell population (Black line histograms: fluorescence of the untreated cells, grey shaded histograms: fluorescence of the cells treated with 20 μ M LY294002 for 15-18 hours). **C:** Fluorescent microscopy imaging of DR5 in SW948 cells in untreated cells and cells exposed with 20 μ M LY294002 for 17 hours, stimulated or not with HGS-TR2J for 15 min. Cells were first pretreated with LY294002 and stained for cell membrane DR5. Next, cells were either kept on ice or treated with HGS-TR2J for 15 min at 37°C before fixation. One representative of at least 3 independent experiments is shown. **D:** DR5-DISC immunoprecipitation in SW948 cells, using the HGS-TR2J antibody. Cells were pretreated or not for 17 hours with 20 μ M LY294002 before stimulation for 15 min using HGS-TR2J and immunoprecipitation with protein G agarose (C: antibody added after cell lysis, T: cells treated with HGS-TR2J). DR5-DISC analysis was performed at least 3 times.

FADD, caspase 8 and c-FLIP. In control cells, 3 caspase 8 forms were detected in the DR5-DISC: the full form, the intermediate p43/41 cleaved form and the p26/24 cleavage product. The latter indicates full cleavage of caspase 8 and therefore release of active caspase 8 from the complex. c-FLIP was mostly present in its intermediate form. In cells pretreated with LY294002, the ratio of each DISC component was similar. No DR4 was found in the DR5-DISC complex, in both LY294002-treated and -untreated cells (data not shown). Later time points (up to 1 hour, when active caspase 8 becomes detectable in whole cell lysates from LY294002-pretreated cells) displayed similar DISC content (data not shown). Thus, the relative proportion of each DISC protein in the DR5-DISC is similar with and without LY294002.

The opposite effects on DR4- and DR5-sensitivity induced by PI3K signaling pathway inhibition are not specific to the SW948/SW948-TR isogenic model

To expand our results to a non-isogenic model, we also assessed the effects of PI3K-specific inhibition with LY294002 on TRAIL receptor-mediated apoptosis in Colo205. These cells were initially highly sensitive to rhTRAIL and to the DR5-targeting antibody and less to the DR4-targeting antibody, indicating that here DR5 was the most potent of the two agonist death receptors. We therefore used concentrations of 50 nM for HGS-ETR1 and 5 nM for HGS-TR2J to induce equivalent amounts of apoptosis via each death receptor. Our results show that LY294002 treatment decreased sensitivity to rhTRAIL

A



B

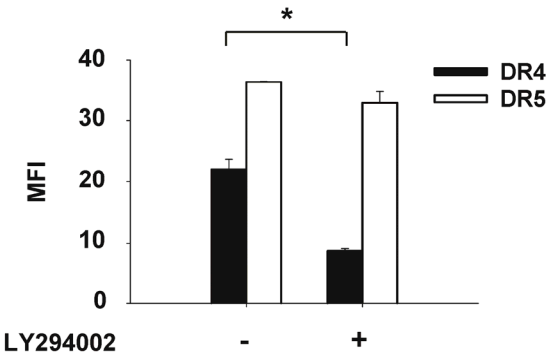


Figure 5: The effects of PI3K inhibition on DR-mediated apoptosis can also be seen in cells that are already more sensitive to HGS-TR2J than to HGS-ETR1 initially. **A:** Sub-G1 content analysis in Colo205. Cells were pre-incubated for 15-18 hours with 20 μ M LY294002, then either left untreated or exposed to rhTRAIL (0.1 μ g/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) for 3 additional hours before harvest. Nuclear DNA was then stained with propidium iodide and the cells analyzed by flow cytometry. The value in percent indicates the amount of apoptosis as measured by the count of sub-G1 events. One representative of at least 3 independent experiments is shown. **B:** Surface expression of TRAIL receptors in Colo205, in untreated cells and after 15-18 hours exposure to 20 μ M LY294002. Receptor expression is measured as Mean Fluorescent Intensity (MFI) normalized to 100.

and HGS-ETR1 (Fig. 5A). Importantly, DR5-mediated sensitivity increased following LY294002 treatment, as seen in SW948 and SW948-TR. Moreover, Colo205 cells also exhibited a reduction in surface membrane DR4 after PI3K inhibition, as seen in SW948 and SW948-TR (Fig. 5B).

Discussion

Prosurvival signals originating from growth factor receptors, including IGF-1R, prevent cell death induced by radiation, cytotoxic drugs and members of the tumor necrosis factor family (21,36-38). In the present study, we demonstrated that inhibition of prosurvival signaling using the IGF-1R inhibitor NVP-AEW541 exclusively increased sensitivity to DR5-mediated apoptosis in SW948 and SW948-TR colon carcinoma cells. SW948 cells in particular were initially very sensitive via DR4, and therefore contained all the proteins necessary to form a DISC, but they exhibited significant resistance to apoptosis mediated via DR5. The IGF-1R inhibitor exclusively released the blockade of caspase 8 cleavage at the level of DR5. The TRAIL-resistant cell line SW948-TR became more sensitive to the anti-DR5 agonistic antibody, suggesting that the effects of IGF-1R inhibition are not restricted to rhTRAIL-sensitive cells. Surprisingly, we found that IGF-1R inhibition decreased sensitivity to rhTRAIL or an anti-DR4 agonistic antibody. The decrease in sensitivity to rhTRAIL and to the anti-DR4 antibody could be explained in part by the ~50% downregulation of membrane DR4 following IGF-1R inhibition seen in all 3 cell lines. We correlated this DR4 downregulation with a decrease in caspase 8 cleavage following rhTRAIL or anti-DR4 treatment. This surprising decrease in sensitivity to some DR-ligand is supported by prior demonstrations that inhibition of

oncogenic pathways such as HER-2 and Ras also reduces the expression of TRAIL receptors, as well as their sensitivity (39,40). Mahalingam *et al.* recently found that inhibition of the c-Jun N-terminal kinase (JNK) stress pathway antagonized TRAIL-induced apoptosis but stimulated anti-DR antibody-induced apoptosis in several colon cancer cells (41). Likewise, a proapoptotic effect of IGF-1 on rhTRAIL sensitivity in 6 out of 9 colon cancer cell lines was described (42). We identified PI3K as the mediator of both DR4 decrease and DR5 increase in sensitivity to their respective agonist antibodies. In our combination study, we used a concentration of NVP-AEW541 above the submicromolar amounts strictly needed to inhibit IGF-1 induced Akt phosphorylation. Although using higher concentrations of NVP-AEW541 might lower its selectivity and inhibit additional receptor tyrosine kinases, inhibiting these additional targets apparently leads to stronger inhibition of the PI3K/Akt pathway. Furthermore, our work using the widely-accepted LY294002 compound clearly indicates a role for PI3K in modulating DR-mediated apoptosis. Thus, our experiments provide a link between the involvement of IGF-1 in PI3K/Akt activity in colon cancer cells and the role of this pathway in death receptor-mediated apoptosis regulation.

We investigated several hypotheses for the increase in DR5-mediated sensitivity following PI3K inhibition. No change in membrane DR5 expression was seen. It has been reported that active PI3K can directly act on the DISC and interfere with caspase 8 cleavage by up-regulating c-FLIP (43). We found that neither basal levels of c-FLIP nor DR5-DISC c-FLIP levels were altered by the PI3K inhibitor in SW948 cells. This indicates that the effects of PI3K on the DR5-DISC are independent of c-FLIP in our model. Similar observations were made regarding the basal levels of other proteins recruited to the DISC. Furthermore, we also excluded that sensitivity to the anti-DR5 antibody following PI3K inhibition increases was caused by receptor aggregation. A number of proteins identified as downstream substrates of PI3K/Akt can modulate the mitochondrial apoptotic pathway of apoptosis, for instance caspase 9 and Bad (44-46). Inhibition of caspase 9 activity using zLEHD-fmk did not protect SW948 cells from apoptosis induced by either TR2J or the combination of TR2J with LY294002, pointing toward a mitochondria-independent mechanism (data not shown). Following LY294002 treatment, both caspase 8 and c-FLIP cleavage was enhanced upon stimulation of DR5 with its agonistic antibody, which strongly supports the existence of a sensitizing mechanism at the DR5-DISC itself. We suggest that PI3K inhibition leads to a higher

turn-over of c-FLIP and caspase 8 at the DR5-DISC, thus gradually increasing the amount of their cleaved cytoplasmic forms.

In Colo205, which is a DR5-driven cell line with regard to the relative potency of each DR to transduce apoptosis (10), sensitivity to the DR5 antibody was remarkably enhanced by LY294002. Conversely, DR4-mediated sensitivity was almost completely lost and sensitivity to rhTRAIL decreased by ~ 50%. Although the downregulation of DR4 could explain part of these effects, the benefits of PI3K inhibition on DR5 signaling seemed restricted to the DR5 antibody but not to rhTRAIL. Thomas *et al.* established (47) that the cytoplasmic tail of DR4 and DR5 had an important role in facilitating FADD binding and DISC formation upon stimulation with DR ligands. Depending on this cytoplasmic tail, the ability of the various DR-ligands to trigger apoptosis differed. Unlike TRAIL, the anti-DR5 agonistic antibody HGS-ETR2 could induce apoptosis independently of the last c-terminal amino-acids of DR5. In view of these findings, we suggest that PI3K inhibition might induce intracellular conformational changes at the level of DR5 that only benefits to HGS-TR2J.

Since PI3K is an essential component in the transduction of survival signals originating from the growth factor receptors, we consider a better understanding of its influence on the TRAIL pathway necessary. Interestingly, while chemotherapy requires the p53 gene to upregulate DR5 and thereby increase death ligand sensitivity in colonic carcinoma (48) PI3K inhibition improved DR5-mediated sensitivity without requiring a functional p53 (the 3 cell lines used in our experiments express mutant p53). Elucidating the mechanism of action of PI3K on DR5 could therefore be decisive for future DR agonist-based therapies, since the vast majority of colorectal cancers carry mutations in p53. Conversely, inhibition of PI3K combined with chemotherapy might further improve sensitivity to DR5 agonists.

DR4 and DR5 can be targeted individually (using antibodies or death-receptor specific forms of TRAIL) or simultaneously (using rhTRAIL), and these ligands will have to be combined with other therapies to overcome resistance mechanisms. We conclude that DR5-targeting agents, such as agonistic antibodies, might be optimal to elicit maximum cell death induction from DR agonist-based regimens combined with drugs inhibiting PI3K.

References

1. Kelly H and Goldberg RM. Systemic therapy for metastatic colorectal cancer: current options, current evidence. *J Clin Oncol* 2005; 23: 4553-4560.
2. Duiker EW, Mom CH, de Jong S, Willemse PH, Gietema JA, van der Zee AG and de Vries EGE. The clinical trail of TRAIL. *Eur J Cancer* 2006; 42: 2233-2240.
3. Kischkel FC, Lawrence DA, Tinel A, LeBlanc H, Virmani A and Schow P. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J Biol Chem* 2001; 276: 46639-46646.
4. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A and Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996; 271: 12687-12690.
5. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA and . Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995; 3: 673-682.
6. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z and Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999; 104: 155-162.
7. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC and Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* 1999; 5: 157-163.
8. Kelley RF, Totpal K, Lindstrom SH, Mathieu M, Billeci K, DeForge L, Pai R, Hymowitz SG and Ashkenazi A. Receptor-selective mutants of apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling. *J Biol Chem* 2005; 280: 2205-2212.
9. MacFarlane M, Kohlhaas SL, Sutcliffe MJ, Dyer MJ and Cohen GM. TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. *Cancer Res* 2005; 65: 11265-11270.
10. van der Sloot AM, Tur V, Szegezdi E, Mullally MM, Cool RH, Samali A, Serrano L and Quax WJ. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. *Proc Natl Acad Sci U S A* 2006; 103: 8634-8639.
11. Chuntharapai A, Dodge K, Grimmer K, Schroeder K, Marsters SA, Koeppen H, Ashkenazi A and Kim KJ. Isotype-dependent inhibition of tumor growth in vivo by monoclonal antibodies to death receptor 4. *J Immunol* 2001; 166: 4891-4898.

12. Griffith TS, Rauch CT, Smolak PJ, Waugh JY, Boiani N, Lynch DH, Smith CA, Goodwin RG and Kubin MZ. Functional analysis of TRAIL receptors using monoclonal antibodies. *J Immunol* 1999; 162: 2597-2605.
13. Ichikawa K, Liu W, Zhao L, Wang Z, Liu D, Ohtsuka T, Zhang H, Mountz JD, Koopman WJ, Kimberly RP and Zhou T. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat Med* 2001; 7: 954-960.
14. Pukac L, Kanakaraj P, Humphreys R, Alderson R, Bloom M, Sung C, Riccobene T, Johnson R, Fiscella M, Mahoney A, Carrell J, Boyd E, Yao XT, Zhang L, Zhong L, von KA, Shepard L, Vaughan T, Edwards B, Dobson C, Salcedo T and Albert V. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types in vitro and in vivo. *Br J Cancer* 2005; 92: 1430-1441.
15. Hague A, Hicks DJ, Hasan F, Smartt H, Cohen GM, Paraskeva C and MacFarlane M. Increased sensitivity to TRAIL-induced apoptosis occurs during the adenoma to carcinoma transition of colorectal carcinogenesis. *Br J Cancer* 2005; 92: 736-742.
16. Koornstra JJ, de Jong S, Hollema H, de Vries EGE and Kleibeuker JH. Changes in apoptosis during the development of colorectal cancer: a systematic review of the literature. *Crit Rev Oncol Hematol* 2003; 45: 37-53.
17. van Geelen CMM, de Vries EGE and de Jong S. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist Updat* 2004; 7: 345-358.
18. Wang S and El Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* 2003; 22: 8628-8633.
19. Cohen SJ, Cohen RB and Meropol NJ. Targeting signal transduction pathways in colorectal cancer--more than skin deep. *J Clin Oncol* 2005; 23: 5374-5385.
20. Cuello M, Ettenberg SA, Clark AS, Keane MM, Posner RH, Nau MM, Dennis PA and Lipkowitz S. Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. *Cancer Res* 2001; 61: 4892-4900.
21. Mitsiades CS, Mitsiades N, Poulaki V, Schlossman R, Akiyama M, Chauhan D, Hideshima T, Treon SP, Munshi NC, Richardson PG and Anderson KC. Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. *Oncogene* 2002; 21: 5673-5683.
22. Park SY and Seol DW. Regulation of Akt by EGF-R inhibitors, a possible mechanism of EGF-R inhibitor-enhanced TRAIL-induced apoptosis. *Biochem Biophys Res Commun* 2002; 295: 515-518.
23. LeRoith D and Roberts CT, Jr. The insulin-like growth factor system and cancer. *Cancer Lett* 2003; 195: 127-137.
24. Baserga R, Peruzzi F and Reiss K. The IGF-1 receptor in cancer biology. *Int J Cancer* 2003; 107: 873-877.
25. Weber MM, Fottner C, Liu SB, Jung MC, Engelhardt D and Baretton GB. Overexpression of the insulin-like growth factor I receptor in human colon carcinomas. *Cancer* 2002; 95: 2086-2095.

26. Hu WH, Johnson H and Shu HB. Tumor necrosis factor-related apoptosis-inducing ligand receptors signal NF-kappaB and JNK activation and apoptosis through distinct pathways. *J Biol Chem* 1999; 274: 30603-30610.
27. Zhou BP, Hu MC, Miller SA, Yu Z, Xia W, Lin SY and Hung MC. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-kappaB pathway. *J Biol Chem* 2000; 275: 8027-8031.
28. Luo J, Manning BD and Cantley LC. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* 2003; 4: 257-262.
29. West KA, Castillo SS and Dennis PA. Activation of the PI3K/Akt pathway and chemotherapeutic resistance. *Drug Resist Updat* 2002; 5: 234-248.
30. Hennessy BT, Smith DL, Ram PT, Lu Y and Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov* 2005; 4: 988-1004.
31. Ihle NT and Powis G. Take your PIK: phosphatidylinositol 3-kinase inhibitors race through the clinic and toward cancer therapy. *Mol Cancer Ther* 2009; 8: 1-9.
32. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M, Hideshima T, Chauhan D, Joseph M, Libermann TA, combining aa, Pearson MA, Hofmann F, Anderson KC and Kung AL. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. *Cancer Cell* 2004; 5: 221-230.
33. van Geelen CMM, de Vries EGE, Le TKP, van Weeghel RP and de Jong S. Differential modulation of the TRAIL receptors and the CD95 receptor in colon carcinoma cell lines. *Br J Cancer* 2003; 89: 363-373.
34. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; 139: 271-279.
35. Pennarun B, Meijer A, de Vries EGE, Kleibeuker JH, Kruyt FA and de Jong S. Playing the DISC: Turning on TRAIL death receptor-mediated apoptosis in cancer. *BBA-Rev Cancer* 2010; 1805: 123-140.
36. Hofbauer S, Hamilton G, Theyer G, Wollmann K and Gabor F. Insulin-like growth factor-I-dependent growth and in vitro chemosensitivity of Ewing's sarcoma and peripheral primitive neuroectodermal tumour cell lines. *Eur J Cancer* 1993; 29A: 241-245.
37. Turner BC, Haffty BG, Narayanan L, Yuan J, Havre PA, Gumbs AA, Kaplan L, Burgaud JL, Carter D, Baserga R and Glazer PM. Insulin-like growth factor-I receptor overexpression mediates cellular radioresistance and local breast cancer recurrence after lumpectomy and radiation. *Cancer Res* 1997; 57: 3079-3083.
38. Wu Y, Tewari M, Cui S and Rubin R. Activation of the insulin-like growth factor-I receptor inhibits tumor necrosis factor-induced cell death. *J Cell Physiol* 1996; 168: 499-509.
39. Drosopoulos KG, Roberts ML, Cermak L, Sasazuki T, Shirasawa S, Andera L and Pintzas A. Transformation by oncogenic RAS sensitizes human colon cells to TRAIL-induced apoptosis by up-regulating death receptor 4 and death receptor 5 through a MEK-dependent pathway. *J Biol Chem* 2005; 280: 22856-22867.

40. Dubska L, Andera L and Sheard MA. HER2 signaling downregulation by trastuzumab and suppression of the PI3K/Akt pathway: an unexpected effect on TRAIL-induced apoptosis. *FEBS Lett* 2005; 579: 4149-4158.
41. Mahalingam D, Keane M, Pirianov G, Mehmet H, Samali A and Szegezdi E. Differential activation of JNK1 isoforms by TRAIL receptors modulate apoptosis of colon cancer cell lines. *Br J Cancer* 2009; 100: 1415-1424.
42. Remacle-Bonnet M, Garrouste F, Baillat G, Andre F, Marvaldi J and Pommier G. Membrane rafts segregate pro- from anti-apoptotic insulin-like growth factor-I receptor signaling in colon carcinoma cells stimulated by members of the tumor necrosis factor superfamily. *Am J Pathol* 2005; 167: 761-773.
43. Panka DJ, Mano T, Suhara T, Walsh K and Mier JW. Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. *J Biol Chem* 2001; 276: 6893-6896.
44. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S and Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 1998; 282: 1318-1321.
45. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997; 91: 231-241.
46. del Peso L, Gonzalez-Garcia M, Page C, Herrera R and Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 1997; 278: 687-689.
47. Thomas LR, Johnson RL, Reed JC and Thorburn A. The C-terminal tails of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas receptors have opposing functions in Fas-associated death domain (FADD) recruitment and can regulate agonist-specific mechanisms of receptor activation. *J Biol Chem* 2004; 279: 52479-52486.
48. Wang S and El Deiry WS. Requirement of p53 targets in chemosensitization of colonic carcinoma to death ligand therapy. *Proc Natl Acad Sci U S A* 2003; 100: 15095-15100.

Sorafenib plus aspirin promotes TRAIL-induced apoptosis by targeting FLIP and Mcl-1 and potentiates growth inhibition in colon cancer cells

Bodvaël Pennarun^{1,2}
Jan H. Kleibeuker²
Wytske Boersma-van Ek²
Frank Kruyt¹
Harry Hollema³
Elisabeth G.E. de Vries¹
Steven de Jong¹

Departments of ¹Medical Oncology, ²Gastroenterology and ³Pathology,
University Medical Center Groningen,
University of Groningen, the Netherlands

Manuscript in preparation

Abstract

The multikinase inhibitor sorafenib is highly effective against certain types of cancer in the clinic and prevents colon cancer cell proliferation *in vitro*. Non-steroidal anti-inflammatory drugs such as acetylsalicylic acid (aspirin) have shown activity against colon cancer cells. The aims of this study were to determine whether the combination of aspirin with sorafenib has enhanced anti-proliferative effects and increases recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL)-induced apoptosis in the human SW948, Lovo, Colo205, Colo320, Caco-2, and HCT116 colon cancer cell lines. In four cell lines, aspirin strongly stimulated the anti-proliferative effects of sorafenib (~ four-fold enhancement) by inducing cell cycle arrest. Furthermore, combining low doses of aspirin (≤ 2.5 mM) and sorafenib (≤ 2.5 μ M) greatly sensitized TRAIL-sensitive and TRAIL-resistant colon cancer cells to rhTRAIL. The increase in rhTRAIL sensitivity was caused by a reduction in basal levels of the anti-apoptotic proteins FLIP and Mcl-1 following aspirin and sorafenib cotreatment, as confirmed with small interfering RNA. Next, the clinical relevance of targeting FLIP and Mcl-1 in colon cancer was examined. Using immunohistochemistry, we found that Mcl-1 expression was significantly increased in colon adenoma and carcinoma patient material compared to healthy colonic epithelium, similar to the enhanced FLIP expression we recently observed in colon cancer. These results underscore the potential of combining low doses of aspirin with sorafenib to inhibit proliferation and target the anti-apoptotic proteins FLIP and Mcl-1. Our findings also support further pre(clinical) investigation of this combination with TRAIL receptor-targeting agents in colon cancer.

Introduction

Colon cancer is a leading cause of cancer-related death in the Western world, accounting for nearly 52,000 death every year in the U.S. only (1). Mortality remains high despite improvements in the area of prevention and treatment. Patients with advanced disease stage might benefit from novel chemotherapeutic options. By inhibiting cyclooxygenase 2 (COX-2) enzymes, non-steroidal anti-inflammatory drugs (NSAIDs) exert anti-inflammatory and anti-pyretic properties. Interestingly, these drugs also exhibit COX-dependent and -independent anti-cancer cell properties (2,3). Although until now clinical trials have failed to show benefits from combining the NSAID celecoxib with chemotherapy in colorectal cancer (4,5), combinations with other NSAIDs and novel chemotherapeutic agents are of potential interest (6).

Sorafenib is a broad spectrum kinase inhibitor targeting the RAF/MEK/ERK pathway and several tyrosine kinase receptors in tumor cells but also the vascular endothelium growth factor (VEGF) receptor 2 and 3 in endothelial cells (7). It is currently applied in the clinic to treat renal cell cancer and hepatocellular cancer. Its use against additional forms of malignancies, including colon cancer, is also being investigated in over 380 studies (source: ClinicalTrials.gov, 2010). Interestingly, some of the indirect targets of sorafenib in cancer cells such as cyclin D1 (8) and Mcl-1 (9) are also targets of aspirin (10,11), suggesting that these drugs could interact with each other in cancer cells through overlapping and/or complementary effects.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a trimeric protein of the tumor necrosis factor superfamily that has the ability to specifically induce apoptosis in cancer cells (12). By binding to trimers comprised of TRAIL receptor 1 (TRAIL-R1) and/or TRAIL-R2 at the cell membrane, TRAIL triggers formation of the so-called death-inducing signaling complex (DISC) which activates the caspase cascade. Therapeutically, the TRAIL receptors are of particular interest for colon cancer since both TRAIL receptor expression and sensitivity to TRAIL increase during colorectal tumor progression (13-15). Phase I clinical studies have demonstrated that TRAIL is well-tolerated in its recombinant human (rh) form, and combination therapies to improve rhTRAIL effectivity are under investigation (16,17).

To increase sensitivity to rhTRAIL in cancer cells, much focus has been placed on combining rhTRAIL with single-drug therapies. Although many agents can sensitize

cells to rhTRAIL *in vitro*, the relevance of such findings is limited due to the high concentrations, often far above the clinically achievable range, needed to induce a pro-apoptotic modulating effect. We therefore chose a different approach and used a combination of two drugs, namely aspirin and sorafenib. Each of these two drugs has already been shown to have potential in combination with rhTRAIL in colon cancer cells. We first studied possible interactions between these two drugs at the molecular level, in colon cancer cell lines. Next, we tested whether combining low doses of aspirin and sorafenib with rhTRAIL would successfully stimulate rhTRAIL-induced apoptosis and determined the intracellular targets involved. Finally, we assessed the clinical relevance of these targets in colon cancer patient material using immunohistochemistry.

Materials and Methods

Reagents

Recombinant human (rh)TRAIL was produced non-commercially following a protocol described earlier (18). The TRAIL-receptor antibodies used for flow cytometry experiments were obtained from Immunex Corporation. 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT)-solution was purchased from Sigma-Aldrich Chemie.

Cell lines

The rhTRAIL-sensitive colon carcinoma cell line SW948 was purchased from the American Type Culture Collection and cultured in Leibovitz L15-Myeloma medium (1:1) enriched with 10 % fetal calf serum (FCS), 0.05 M pyruvate, 0.1 M glutamine and 0.025 % β -mercaptoethanol at 37 °C in a humidified atmosphere with 5 % CO₂. Colo205, Colo320 and Lovo colon carcinoma cell lines were purchased from the ATCC and grown in RPMI containing 10 % FCS at 37 °C in a humidified atmosphere with 5 % CO₂. The Caco-2 colon carcinoma cell line was purchased from the ATCC and grown in RPMI containing 13 % FCS at 37 °C, in a humidified atmosphere with 5 % CO₂. HCT116 cells were kindly provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD) and cultured in McCoy's 5A medium supplemented with 10 % FCS in a humidified atmosphere at 37 °C with 5 % CO₂.

Cytotoxicity assay

A microculture tetrazolium (MTT) assay was used to determine cytotoxicity. Cells were incubated in a total volume of 200 μ L. After an incubation period of 96 h, 20 μ L of 5 mg/ml MTT solution diluted in PBS (6.4 mM Na_2HPO_4 ; 1.5 mM KH_2PO_4 ; 0.14 mM NaCl; 2.7 mM KCl; pH=7.2) was added for 3.75 h. Subsequently, plates were centrifuged and the supernatant aspirated. After dissolving the formazan crystals by adding dimethyl sulfoxide (Merck, Amsterdam, The Netherlands), plates were read immediately at 520 nm using a microtiter well spectrometer (Bio-Rad microplate reader). Controls consisted of media without cells. Cell survival was defined as the growth of treated cells compared to untreated cells. Mean cytotoxicity was determined in three independent experiments where each condition was performed in quadruplicate.

Flow cytometry

Analysis of TRAIL-receptor membrane expression was performed with a flow cytometer (Epics Elite, Coulter Electronics), and cells were stained as described previously (18). The following antibodies were used: huTRAILR1-M271 for DR4, huTRAILR2-M413 for DR5 (both were a gift from Amgen). Membrane expression was measured as the increase in mean fluorescence intensity (MFI) of the whole analyzed cell population.

Apoptosis assay

For each cell line 5×10^3 cells were seeded in 96-well plates and after attachment cells were treated with appropriate reagents for the desired courses. Apoptosis was assessed by staining nuclear chromatin with acridine orange and identification of the morphological changes by fluorescence microscopy. Apoptosis was determined by counting the number of apoptotic cells in a total of 300 cells and expressed as percentage apoptotic cells. All apoptosis assays were performed in triplicate and at least three independent assays were performed.

SDS-polyacrylamide gel electrophoresis and Western blotting

Preparation of protein lysates and Western blot analysis were performed as described previously (18). The following antibodies were used: mouse anti-FADD from Transduction Laboratories, rabbit anti-phosphorylated pRB (Ser780), mouse anti-

caspase 8, rabbit anti-caspase 9, anti-caspase 3, anti-cleaved caspase 3 and anti-Bak from Cell Signaling Technology, mouse anti-actin from ICN Biomedicals, mouse-anti-FLIP NF6 from Alexis, mouse anti-c-myc, rabbit anti-cyclin D1 from Santa Cruz. Mouse anti-Mcl-1 was purchased from Calbiochem. Rabbit anti-poly ADP ribose phosphate was from Roche. The horseradish peroxidase-labeled secondary antibodies were from DAKO, and chemiluminescence was detected using the BM-chemiluminescence kit or with the Lumi-Light Plus Western blotting kit (Roche Diagnostics). Protein concentrations were determined using the DC Biorad protein assay. In all experiments 20-40 µg protein lysates were loaded on gel. Actin expression was used as loading control. Western blot analyses were performed at least three times.

BrdU/propidium iodide cell cycle staining

Cells were seeded in 6-well plates, treated for the indicated times before harvest. Cells were then stained using the BrdU/FITC flow kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. Propidium iodide was substituted to 7-AAD for total DNA staining before analysis on a FACSCalibur flow cytometer (BD Biosciences). Three independent assays were performed.

Transfection with siRNA

For small interfering RNA (siRNA)-mediated downregulation of the various mRNA of interest using the first set of duplexes, the following PAGE-purified siRNA sequences were purchased from Eurogentec: 5'-GAGGUAAGCUGUCUGUCGGdTdT-3' (sense strand) and 5'-AUGGGCCUCCGGUUCAUGCdTdT-3' (anti-sense strand) for FLIP, (5'-GAAACGCGGUAAUCGGACUdTdT-3' (sense) and 5'-AGUCCGAUUACCGCGUUUCdTdT-3' (anti-sense) for Mcl-1 (19), 5'-UGAGUACUUCACCAAGAUUdTdT-3' (sense) and 5'-AAUCUUGGUGAAGUACUCAdTdT-3' (anti-sense) for Bcl-2 homologous antagonist/killer 1 (Bak). A second set of siRNA was also used: 5'-CATCCACAGAATAGACCTGdTdT-3' (sense strand) and 5'-CAGGTCTATTCTGTGGATGdTdT-3' (anti-sense strand) for FLIP (20), 5'-GGTCGGGGAATCTGGTAATdTdT-3' (sense) and 5'-ATTACCAGATTCCCCGACCDdTdT-3' (anti-sense) for Mcl-1 (19), 5'-GAGUACAGAAGCUUUAGCAdTdT-3' (sense) and 5'-UGC UAAAGCUUCUGUACUCdTdT-3' (anti-sense) for Bak (home-made). A scrambled sequence without any known homology to the human genome was used as negative control siRNA, also from

Eurogentec. Cells were transfected in 96-well (5,000 cells/well) or 6-well plates (0.3×10^6 cells/well) with 0.5 or 20 μ L respectively of 20 μ M siRNA duplexes with Lipofectamine 2000 reagent, following the manufacturer's instructions (Invitrogen). The same absolute amounts of siRNA were used for double siRNA transfections. To maintain the RNA/Lipofectamine 2000 ratio and the amounts of each individual siRNA between experiments, single siRNA conditions were equalized to co-siRNA transfection conditions by mixing single targeted siRNA with scrambled siRNA, at a 1:1 ratio. The next day, cells were treated with various drugs for the indicated times before apoptosis determination using acridine orange staining (96-well plate) and/or protein isolation for Western-blotting (6-well plate). All apoptosis assays were performed in triplicate and at least three independent assays were performed.

TaqMan real-time PCR

Total RNA was isolated with the Qiagen RNeasy mini kit using the manufacturer's instructions. Trace amounts of DNA contamination were removed by DNase I digestion. cDNA was reverse-transcribed into cDNA from 1600 ng purified RNA using MMLV reverse transcriptase and oligo (dT)11 primers, as described by the manufacturer's protocol (Life Technologies). The profile of the reverse transcription reaction was 10 min at 70 °C, 50 min at 42 °C, and 10 min at 70 °C. Taqman PCR (qRT-PCR) was performed on 50 ng of cDNA. Applied Biosystems Taqman Gene expression assays and Taqman Universal PCR master mix were used to perform qRT-PCR on FLIP (Hs01116280_m1, Applied Biosystems), Mcl-1 (Hs01050896_m1, Applied Biosystems) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs02758991_g1, Applied Biosystems). All reactions were performed in triplicate in 384-well plates and measured on an ABI PRISM 7900 HT Sequence Detection System. Amplification of the samples was carried out in triplicate in a final reaction volume of 20 μ L containing 10 μ L 2X PCR Mix, 4 μ L H₂O, 1 μ L of each assay on demand (5 μ M) and 5 μ L cDNA (1:3). PCR reaction conditions were as follows: Step 1: AmpErase uracil-N-glycosylase incubation for 2 min at 50 °C, step 2: 10 min AmpliTaq Gold activation at 95 °C, step 3: 50 cycles of 15 s denaturation at 95 °C followed by annealing/extension at 60 °C for 1 min. Presence of unique reaction products was determined from the melting curves obtained at the end of 50 cycles of amplification.

To determine RT-PCR efficiency and initial starting quantity of the samples, a standard curve was generated using a 1:3 serial dilution from total starting cDNA sample. Water control and samples without reverse transcription were included to check for absence of DNA contamination. Differences in the amount of starting cDNA between samples were corrected using GAPDH as a housekeeping reference gene. Experiments were performed with cDNA generated from four independent RNA isolation procedures.

Mcl-1 immunohistochemistry staining

Selection of patient and tissue material was described earlier (21). A small number of samples from the original report had to be excluded because of insufficient remaining material for further analysis. Immunohistochemistry was performed on samples embedded in paraffin using the benchmark system (Ventana). Fresh serial 3 μ m thick sections were cut from paraffin blocks. Following deparaffinization in xylol and rehydration in alcohol, antigen retrieval was performed by immersing the slides in 10 mM citrate buffer pH 6 for 15 min in a microwave. After blocking of endogenous peroxidase with H₂O₂, rabbit anti-Mcl-1 (1:400) antibody (Pharmingen) was applied for 1 h at room temperature. Binding specificity was controlled using rabbit IgG1 isotype control (Dakocytomation). Aspecific binding for both antibodies was prevented by adding 1 % standard human serum to the antibody dilution solution (PBS). After several washing steps, the Envision horseradish peroxidase system was used according to the manufacturer's instructions (DAKO). Peroxidase activity was visualized with diaminobenzidine. All slides were counterstained with hematoxylin. Slides were evaluated by an experienced pathologist (HH) and two investigators (BP and WB), using an Olympus BX50 microscope. Expression was quantified semi-quantitatively according to the following scale: negative (less than 10 % positive cells), moderate (+), strong (++) and very strong (+++) based on the intensity of the perinuclear staining. In case of heterogeneous staining, the highest staining intensity was chosen when found in >50 % of the tumor. When the observers' score differed, the smaller value was selected to avoid over-estimating the staining intensity. Diffuse expression was very weak and mostly detected in epithelial cells near the luminal surface in healthy control, as reported by Krajewski *et al.* (22). For statistical analysis, all classes were afterwards

dichotomized. Moderate, strong and very strong staining were considered positive and negative staining as negative.

Statistical analysis

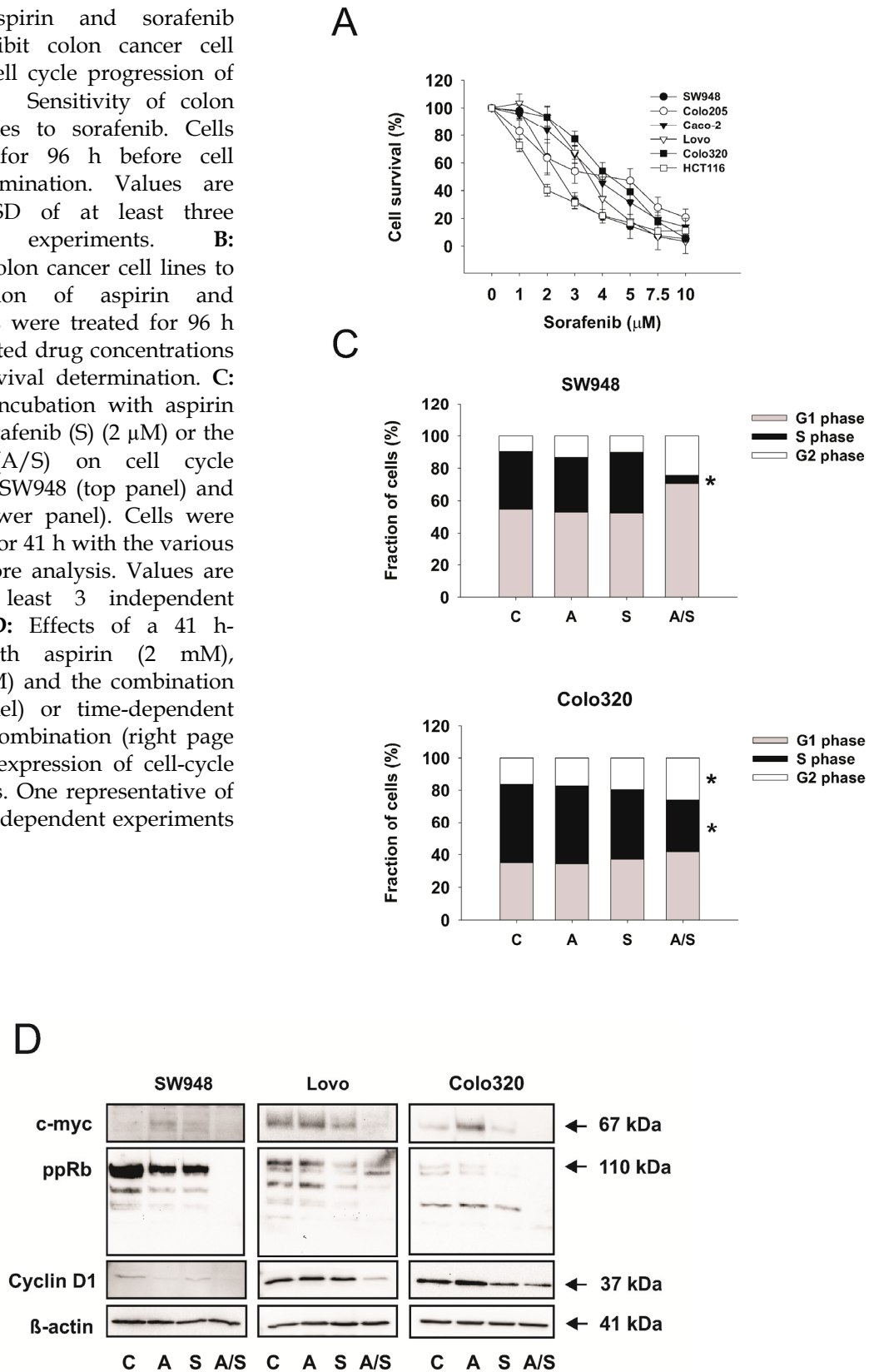
Data are represented as the mean \pm SD. In all cases, statistical analyses were done using 2-tailed student's *t* test. *P*-values < 0.05 were considered significant and are indicated with an asterisk. In the immunohistochemical study, differences in Mcl-1 expression between normal, adenoma and carcinoma were assessed using Chi-square tests. *P*-values < 0.05 were considered significant.

Results

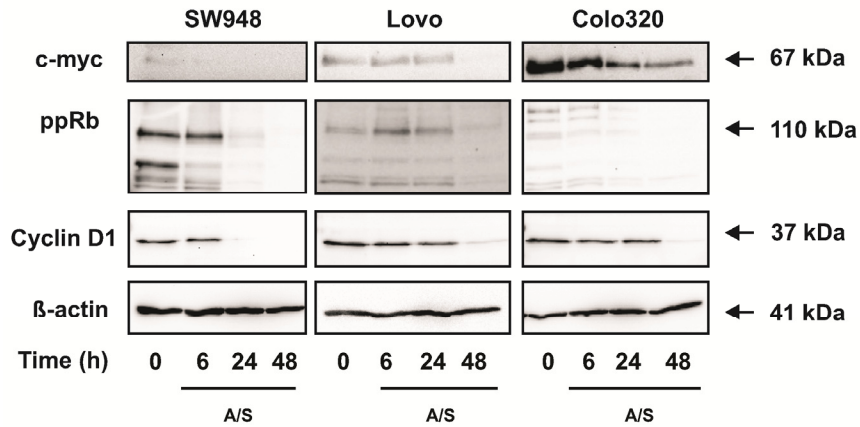
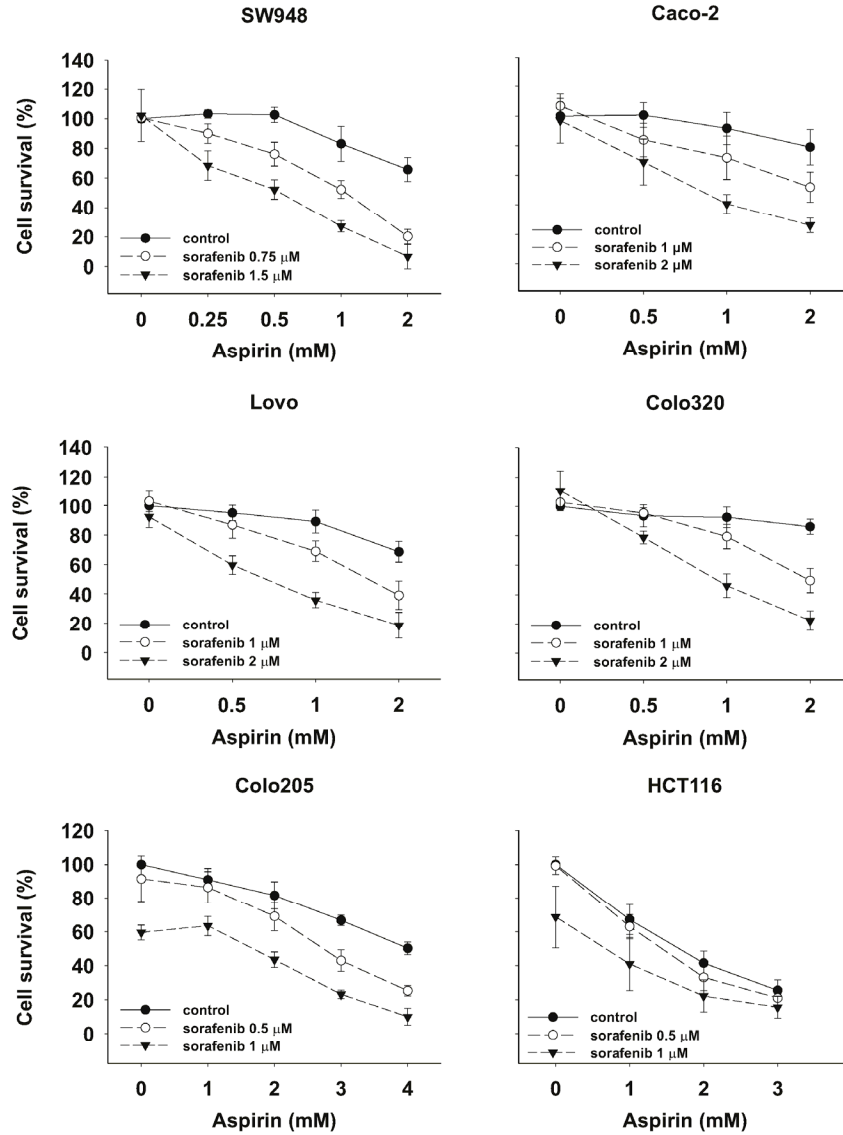
The combination of aspirin and sorafenib potently inhibits colon cancer cell growth

Sorafenib was previously shown to possess anti-proliferative activity in colon cancer cell lines (7,8). First, we determined in a 96-h viability assay the cell growth inhibitory effect of sorafenib in our panel of human colon cancer cell lines, composed of SW948, Lovo, Colo205, Colo320, Caco-2 and HCT116. All cell lines tested showed micromolar sensitivity to the compound, with an average concentration of sorafenib necessary to inhibit growth by 50% of less than 7.5 μ M (GI₅₀; the drug concentration causing a 50% reduction in cell growth compared to the untreated cells) (Fig. 1A, see also Supplementary Fig. S1 for exact GI₅₀ values). This GI₅₀ value is within reported plasma concentrations for sorafenib (23). The GI₅₀ of SW948, Lovo, Colo320 and HCT116 cells were comparable or even below the GI₅₀ values of the B-Raf mutant cell lines Caco-2 and Colo205 (V600E) (24,25)(see also <http://www.sanger.ac.uk>). All cell lines also showed sensitivity to aspirin alone (Supplementary Fig. S2). Next, we show that in 4 colon cell lines, namely, SW948, Lovo, Colo320, and Caco-2, the effects of sorafenib were potentiated by aspirin (Fig. 1B). In Colo205 and HCT116 cells, the effects of the combination on cell survival were mostly additive. Aspirin increased the growth inhibitory effects of sorafenib in B-Raf wild-type SW948, Lovo and Colo320 cells, which suggests that the response is independent of the presence of activating mutations in B-Raf.

Figure 1: Aspirin and sorafenib effectively inhibit colon cancer cell survival and cell cycle progression of these cells. **A:** Sensitivity of colon cancer cell lines to sorafenib. Cells were treated for 96 h before cell survival determination. Values are mean \pm SD of at least three independent experiments. **B:** Sensitivity of colon cancer cell lines to the combination of aspirin and sorafenib. Cells were treated for 96 h with the indicated drug concentrations before cell survival determination. **C:** Effects of an incubation with aspirin (A) (2 mM), sorafenib (S) (2 μ M) or the combination (A/S) on cell cycle distribution in SW948 (top panel) and in Colo320 (lower panel). Cells were pre-incubated for 41 h with the various treatments before analysis. Values are mean of at least 3 independent experiments. **D:** Effects of a 41 h-incubation with aspirin (2 mM), sorafenib (2 μ M) and the combination (left page panel) or time-dependent effects of the combination (right page panel) on the expression of cell-cycle related proteins. One representative of at least three independent experiments is shown.



B



Combining aspirin and sorafenib inhibits cell cycle progression in colon cancer cells

To identify the cause of the inhibition of cell growth by aspirin plus sorafenib, we investigated the effects of aspirin, sorafenib and the combination on cell cycle distribution in SW948 and Colo320 cells (Fig. 1C). In both cell lines, simultaneous treatment with aspirin and sorafenib significantly decreased the percentage of cells in S phase compared to control, from 49.2 to 32.5% in Colo320 ($P < 0.01$) and from 35.3 to 5.1% in SW948 ($P < 0.005$). Colo320 cells also exhibited a significant increase in percentage of cells in G₂/M, from 26.3 to 32.3% ($P < 0.05$). Similar results were found in Lovo cells where aspirin and sorafenib co-treatment decreased the percentage of cells in S phase and increased the percentage of cells in G₂/M compared to control, from 18.2 to 7.9% ($P < 0.01$) and from 38.9 to 51.9% ($P < 0.05$), respectively (Supplementary Fig. S3). This suggests that the decrease in cell survival following co-treatment with aspirin and sorafenib is due to inhibition of cell cycle progression. Next, we assessed the expression of several cell cycle proteins that have been individually reported as targets of aspirin and sorafenib. At the concentrations used, single treatment with aspirin or sorafenib did not alter the expression levels of the cell cycle protein cyclin D1 and phosphorylated pRB (ppRB) or of the oncoprotein c-myc, as shown in three colon cancer cell lines (Fig. 1D, left panel).

Combination of aspirin and sorafenib however led to important decreases in expression of proteins involved in regulating progression through the G₁ phase of the cell cycle such as cyclin D1, ppRb and c-myc, as seen consistently in all three cell lines. The effects were time-dependent (Fig. 1D, right panel) and a striking reduction in expression was seen after 24 to 48 h incubation with aspirin and sorafenib, depending on the cell line. Altogether, our results suggest that the combination of aspirin and sorafenib induces cell cycle arrest.

Low doses of aspirin and sorafenib synergize to increase TRAIL sensitivity

The anti-apoptotic proteins FLIP and Mcl-1 have been individually reported as targets of aspirin and sorafenib. We noticed that the expression of both proteins was much more efficiently inhibited by the combination of aspirin and sorafenib (Fig. 2A, left panel). The decrease in FLIP and Mcl-1 was seen across cell lines, and was time-dependent (Figure 2A, right panel). Both FLIP and Mcl-1 have been associated with resistance to TRAIL in cancer cells, at different levels of the apoptotic signalling. Notably, FLIP can inhibit

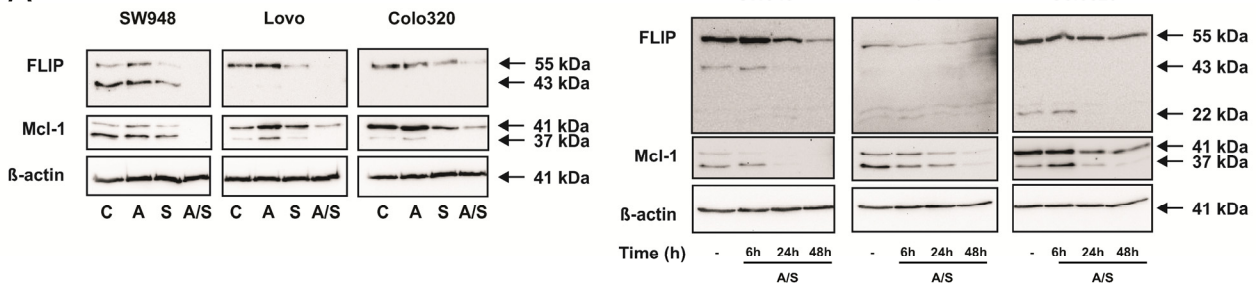
DISC formation and caspase 8 cleavage (26), while Mcl-1 inhibits the mitochondrial route of apoptosis by sequestering pro-apoptotic Bak (27).

Previously, we reported the strong resistance of Colo320 cells and limited sensitivity of Lovo cells to the pro-apoptotic agent rhTRAIL (15,18). Because aspirin and sorafenib decreased expression of the anti-apoptotic proteins FLIP and Mcl-1, we tested whether rhTRAIL sensitivity was increased. In Lovo and Colo320 cells, limited apoptosis ($\leq 30\%$) was induced by a 5 h-rhTRAIL treatment (100 ng/mL) if cells were only pretreated with aspirin and/or sorafenib for 17 h (Figure 2B, left panel). Because we showed that optimal FLIP and Mcl-1 downregulation required long incubation with aspirin and sorafenib, we extended the pre-incubation with these two drugs from 17 to 36 h before rhTRAIL treatment. Following 36 h pre-incubation both with aspirin and sorafenib, rhTRAIL (100 ng/mL) sensitivity markedly increased in Lovo and Colo320 cells, up to about 60 and 40%, respectively (Fig. 2B, right panel). In Lovo and in Colo320 cells, aspirin, sorafenib or their combination did not induce apoptosis. In Lovo cells, aspirin also slightly enhanced rhTRAIL-induced apoptosis ($\leq 40\%$) but less efficiently than the combination of all three therapeutics.

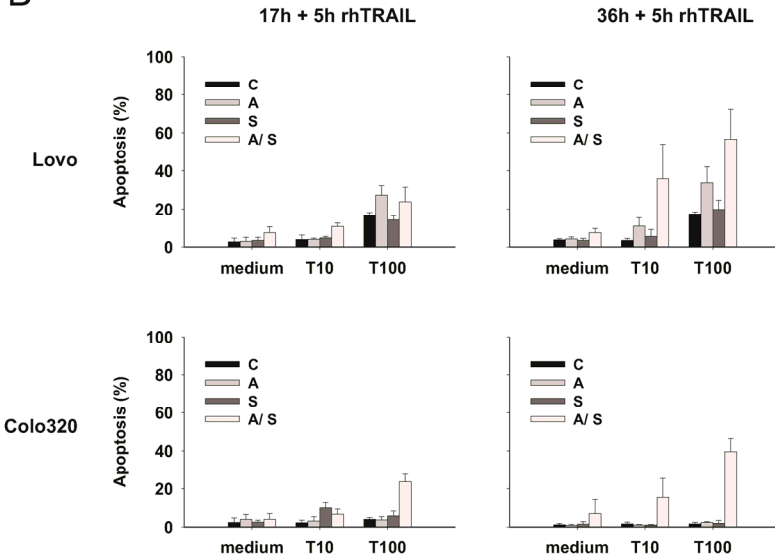
We questioned whether longer treatment with rhTRAIL (17 h) could induce higher levels of apoptosis within the same total treatment window (41 h). When cells were pre-treated with aspirin and sorafenib for 24 h before treatment with rhTRAIL (10-100 ng/mL) for an additional 17 h, apoptosis levels could be further increased up to 80% in Lovo and 70% in Colo320 cells (compare Fig. 2C with 2B, right panel). In both cell lines, apoptosis was greatly stimulated by relatively low amounts of rhTRAIL (10 ng/mL) in combination with aspirin and sorafenib.

Next, we analyzed the effects of aspirin, sorafenib, rhTRAIL and their combination on activation of proteins of the TRAIL apoptotic pathway in Lovo (Fig. 2D, left panel) and Colo320 cells (Fig. 2D, right panel). Treating Lovo cells with aspirin, sorafenib or a combination of these drugs for 41 h induced (slight) cleavage of caspase 9 only. Treatment with rhTRAIL alone (10 ng/mL) for the last 17 h of incubation slightly stimulated caspase 9 and PARP cleavage. Pre-incubation with aspirin or sorafenib did not further induce cleavage of these proteins upon rhTRAIL treatment. However, when cells were pre-treated with both aspirin and sorafenib, potent processing of caspase 8, Bid, caspase 9, caspase 3 and PARP was seen in response to rhTRAIL, indicating high levels of apoptosis. In Colo320 cells as well, only the combination of rhTRAIL, aspirin

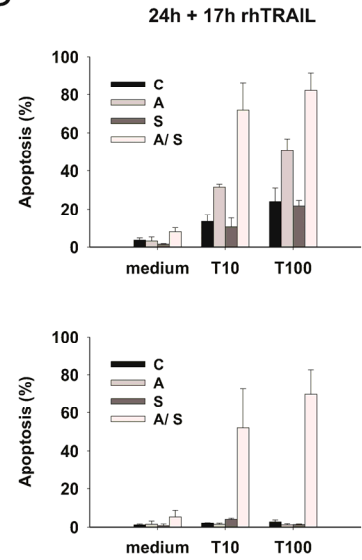
A



B



C



D

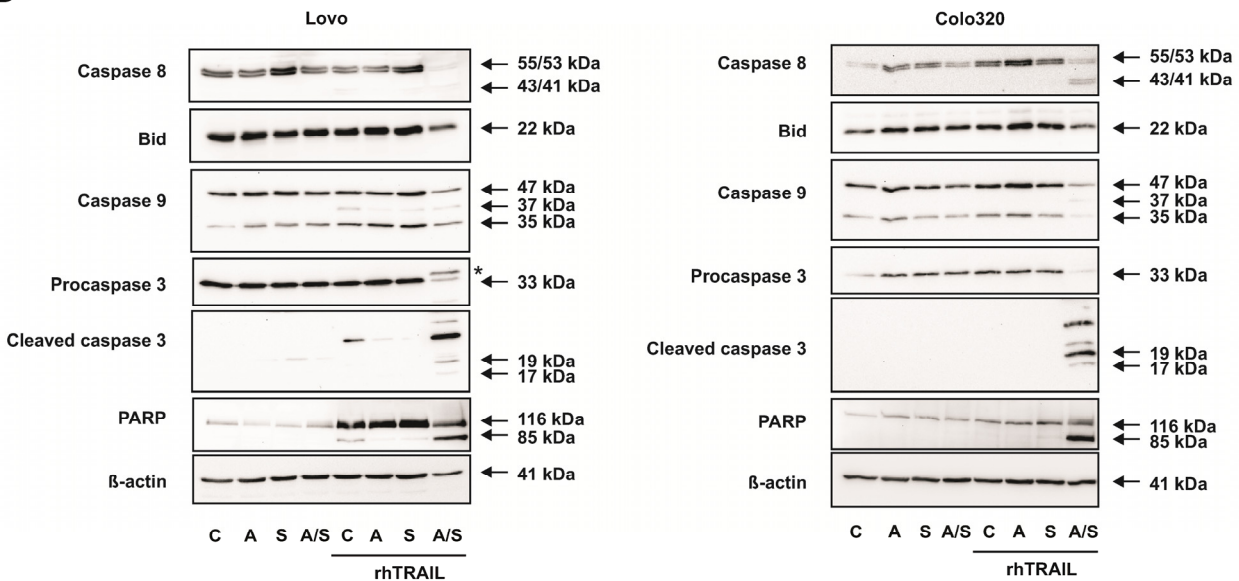


Figure 2: The combination of aspirin and sorafenib downregulates anti-apoptotic proteins and sensitize colon cancer cells to rhTRAIL-induced apoptosis. **A:** Effects of a 41 h-incubation with aspirin (2 mM), sorafenib (2 μ M) and the combination (left panel) or time-dependent effects of the combination (right panel) on the expression of anti-apoptotic proteins. One representative of at least three independent experiments is shown. **B:** Apoptosis in cells pre-incubated with aspirin (2 mM), sorafenib (2 μ M) or the combination before treatment with 10-100 ng/ml rhTRAIL (indicated as T10 and T100, respectively) in Lovo and Colo320 cells. Cells were pre-incubated for 17 (left) or 36 h (right) with aspirin and/or sorafenib before treatment with rhTRAIL (T) for 5 h. **C:** Apoptosis in cells pre-incubated for 24 h with aspirin (2 mM), sorafenib (2 μ M) or the combination before treatment with rhTRAIL for 17 h in Lovo and Colo320 cells. **D:** Western-blot analysis showing activation of caspases, Bid and PARP in Lovo (left) and Colo320 (right) cells pre-treated with aspirin (2 mM) and/or sorafenib (2 μ M) for 24 h before 17 h-treatment with rhTRAIL (10 ng/ml).

and sorafenib could induce cleavage of caspase 8, Bid, caspase 9, caspase 3 and PARP. Similar results were seen in the SW948 cell line (Supplementary Fig. S4). These results confirm that the combination of rhTRAIL, aspirin and sorafenib achieve levels of apoptosis superior to combinations of two of these agents only.

The decrease in Mcl-1 and c-FLIP expression levels is involved in the effects of aspirin and sorafenib on rhTRAIL-induced apoptosis

We then investigated whether the aspirin and sorafenib-induced decrease in FLIP and Mcl-1 protein levels caused the increase in rhTRAIL sensitivity. The expression of these proteins was therefore selectively inhibited using a small interfering RNA (siRNA) approach in Lovo cells. For each protein, efficient expression silencing was confirmed with two different sets of sequences by Western-blot (Fig. 3A, top). Following downregulation of FLIP or Mcl-1 with the each set of siRNAs, cells were stimulated with rhTRAIL (Fig. 3A, bottom). Results show that rhTRAIL sensitivity increased, confirming the ability of FLIP and Mcl-1 to inhibit TRAIL receptor-mediated apoptosis.

Next, we investigated using Western-blotting which steps of the rhTRAIL-induced apoptotic pathway were affected by FLIP and Mcl-1 (Fig. 3B). FLIP downregulation stimulated procaspase 8 processing in presence of rhTRAIL in Lovo cells. As expected, Mcl-1 downregulation did not increase caspase 8 cleavage in response to rhTRAIL. Cleavage of procaspase 3 and PARP were enhanced by FLIP and Mcl-1 siRNA but not cleavage of procaspase 9. These findings indicate that both FLIP and Mcl-1 downregulation enhanced rhTRAIL-induced apoptosis.

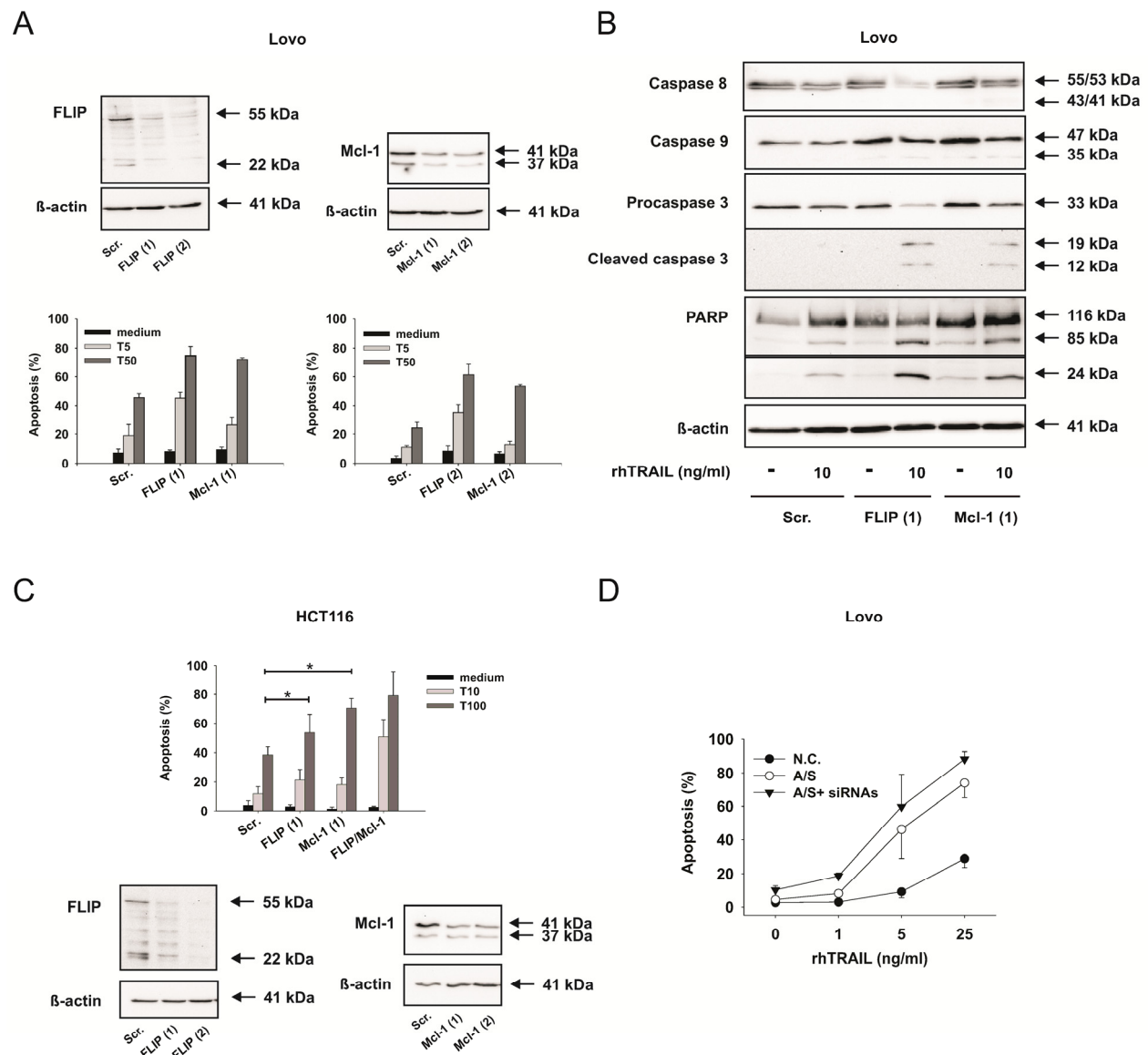


Figure 3: FLIP and Mcl-1 downregulation sensitize colon cancer cells to rhTRAIL-induced apoptosis. **A:** Downregulation of FLIP or Mcl-1 in Lovo cells using siRNA sensitizes cells to rhTRAIL. Cells were either transfected with scrambled siRNA (Scr.) or with two sets of FLIP- or Mcl-1-specific siRNA for 32 h before treatment with 5 and 50 ng/ml rhTRAIL (T5 and T50, respectively) for 17 h. **B:** Activation of caspases and PARP in siRNA-transfected Lovo cells treated with rhTRAIL for 17 h. Cells were transfected for 32 h with negative control or specific siRNAs before treatment with rhTRAIL (10 ng/ml) for 17 h. **C:** Apoptosis in HCT116 cell transfected with negative control, FLIP or Mcl-1 siRNA for 24 h before treatment with rhTRAIL 10 and 100 ng/ml rhTRAIL (indicated as T10 and T100, respectively) for 24 h. Western blots demonstrating the efficacy of the two siRNA sets specific for FLIP or Mcl-1 in HCT116 are also shown. **D:** Effects of aspirin and sorafenib co-treatment on rhTRAIL-induced apoptosis in cells transfected with FLIP and Mcl-1 siRNAs. Apoptosis was determined in Lovo cells treated for 17 h with rhTRAIL following prior transfection for 24 h with scrambled siRNA, with both FLIP and Mcl-1 siRNAs, or with both FLIP and Mcl-1 siRNAs simultaneously to aspirin (2 mM) and sorafenib (2 μ M) co-treatment.

Colo320 cells were refractory to transient siRNA transfection (data not shown). We therefore used HCT116 cells to confirm the importance of FLIP and Mcl-1 levels for rhTRAIL-sensitivity in colon cell lines. These cells were also sensitized to rhTRAIL with a combination of aspirin and sorafenib (data not shown). Downregulating FLIP, Mcl-1 or both proteins increased rhTRAIL-induced apoptosis in HCT116 cells, thus supporting the importance of FLIP and Mcl-1 for rhTRAIL sensitivity in colon cancer cells (Fig. 3C). To further investigate the contribution of FLIP and Mcl-1 downregulation to the effects on rhTRAIL sensitivity of the combination of aspirin with sorafenib, we combined siRNA downregulation of FLIP and Mcl-1 together with aspirin and sorafenib co-treatment before incubation with rhTRAIL (Fig. 3D). In cells transfected with scrambled siRNA, rhTRAIL (25 ng/mL) induced apoptosis in ~30% of Lovo cells. RhTRAIL treatment induced apoptosis in up to 70% of cells following co-downregulation of FLIP and Mcl-1. Combining aspirin and sorafenib co-treatment shortly after transfection with FLIP and Mcl-1 siRNA induced some minor toxicity in absence of rhTRAIL, but it only slightly increased rhTRAIL sensitivity compared to cells with FLIP and Mcl-1 siRNAs only. Although aspirin and sorafenib might modulate the expression of additional pro- or anti-apoptotic proteins, this indicates that downregulation of FLIP and Mcl-1 plays a major role in the increase in rhTRAIL sensitivity.

We investigated whether the diminished FLIP and Mcl-1 protein levels were due to a decrease in mRNA levels (Supplementary Fig. S5). Quantitative real time PCR indicates that aspirin and sorafenib, either alone or in combination, did not decrease FLIP or Mcl-1 mRNA levels in Colo320 or Lovo. The decrease in FLIP and Mcl-1 protein levels following incubation with aspirin and sorafenib is likely caused by either decreased translation or lower stability of these proteins.

RhTRAIL-induced apoptosis following Mcl-1/ FLIP downregulation or aspirin/sorafenib pre-incubation requires activation of the mitochondrial pathway

To investigate the increase in TRAIL sensitivity following Mcl-1 downregulation in relation to the mitochondrial apoptotic pathway, we inhibited Bcl-2 homolog killer 1 (Bak) expression in Lovo cells. Since Lovo cells do not express Bax (28), depleting Bak should impair activation of the mitochondrial apoptotic pathway (29,30). Bak was efficiently downregulated (Fig. 4A, left) and completely blocked rhTRAIL-induced apoptosis (Fig. 4A, middle), suggesting that Lovo is a type II cell line (31). The increase

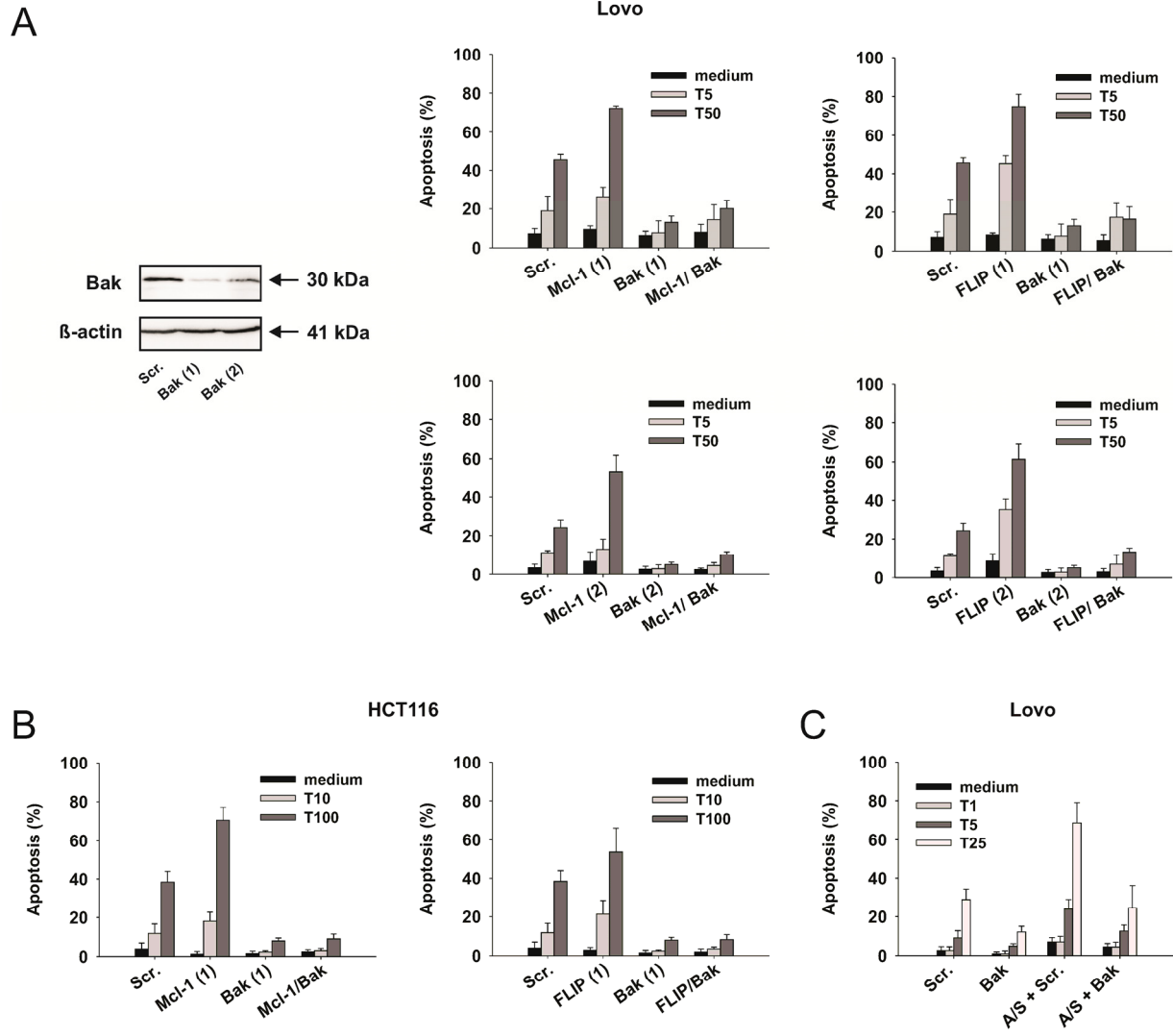


Figure 4: Mcl-1 downregulation, FLIP downregulation and the combination aspirin/sorafenib sensitizes Lovo and HCT116 cells to rhTRAIL-induced apoptosis via the mitochondria. **A:** Bak downregulation prevents rhTRAIL-induced apoptosis in Lovo cells, co-transfected or not with FLIP or Mcl-1 siRNA. Cells were either transfected with scrambled siRNA or with two different Bak-specific siRNAs before harvest and Western-blotting (left panel). Apoptosis in Lovo cells transfected with negative control, Mcl-1, Bak or a combination of Mcl-1 and Bak siRNAs 32 h before rhTRAIL treatment for 17 h (middle panel). Alternately, Lovo cells were transfected with scrambled, FLIP, Bak or a combination of FLIP and Bak siRNA 32 h before treatment with rhTRAIL for 17 h (right panel). Results obtained with two different sets of siRNAs specific for Bak, FLIP and Mcl-1 are shown. **B:** Apoptosis in HCT116 cells transfected with scrambled, Mcl-1, Bak or a combination of Mcl-1 and Bak siRNAs 32 h before rhTRAIL treatment for 17 h (left figure). HCT116 cells were also transfected with scrambled, FLIP, Bak or a combination of FLIP and Bak siRNAs 32 h before treatment with rhTRAIL for 17 h (right figure). **C:** The mitochondrial route plays an important role for rhTRAIL-induced apoptosis in Lovo cells co-treated with aspirin and sorafenib. Lovo cells were transfected with scrambled and/or Bak siRNA for 24 h, alone or in combination with simultaneous aspirin/sorafenib treatment followed by a 17 h-incubation with rhTRAIL at the indicated concentrations.

in rhTRAIL sensitivity following Mcl-1 downregulation required Bak, consistent with the established role of Mcl-1 as a modulator of the intrinsic pathway of apoptosis (Fig. 4A, middle). Interestingly, the pro-apoptotic effect of FLIP downregulation on TRAIL-induced apoptosis was also lost when Bak was also downregulated (Fig. 4A, right). These findings were confirmed with a second set of FLIP, Mcl-1 and Bak siRNAs (Fig. 4A, lower panel). We obtained similar results in the Bax and Bak-expressing HCT116 cell line (Fig. 4B). Inhibition of Bak expression alone prevented basal rhTRAIL sensitivity in control HCT116 cells but also rhTRAIL sensitivity in cells sensitized with siRNA targeting FLIP or Mcl-1. Although in some cell lines downregulating FLIP before rhTRAIL treatment converts type II cells into type I cells (32), this does not seem to be sufficient in Lovo and HCT116 cells.

Next, we tested whether the effects of aspirin and sorafenib cotreatment on rhTRAIL-induced apoptosis was also mediated by the mitochondrial pathway of apoptosis in a Bak-dependent manner (Fig. 4C). In vehicle-treated cells, rhTRAIL induced apoptosis in up to 30% of Lovo cells, which was inhibited by Bak downregulation. Treatment with aspirin and sorafenib greatly increased rhTRAIL-induced apoptosis in cells transfected with scrambled siRNA. The pro-apoptotic effect was lost in Bak-suppressed cells. These results further strengthen our finding that, by downregulating FLIP and Mcl-1, aspirin and sorafenib sensitizes cells to rhTRAIL in a mitochondria-dependent manner.

FLIP and Mcl-1 expression increases during colon carcinogenesis

Studies performed previously in our lab using patient material demonstrated a significant increase in FLIP protein levels in colorectal cancer compared to normal colon mucosa and colon adenoma (21). The therapeutic relevance of Mcl-1 protein in colon cancer has not been clearly established. Mcl-1 expression was therefore examined in the patient material that we previously used to evaluate FLIP expression (Fig. 5A). Altogether, Mcl-1 staining was detected in 70.5% of all adenomas ($n = 57$) and 59.1% of all carcinomas ($n = 49$), which was significantly higher than the 10% positivity found in normal mucosa ($n = 20$) (both $P < 0.001$) (Fig. 5B). Staining of Mcl-1 was perinuclear, which has been associated with an anti-apoptotic function at the mitochondria (33). Thus, our results highlight the potential of Mcl-1 as a marker and/or therapeutic target in colon adenoma and carcinoma.

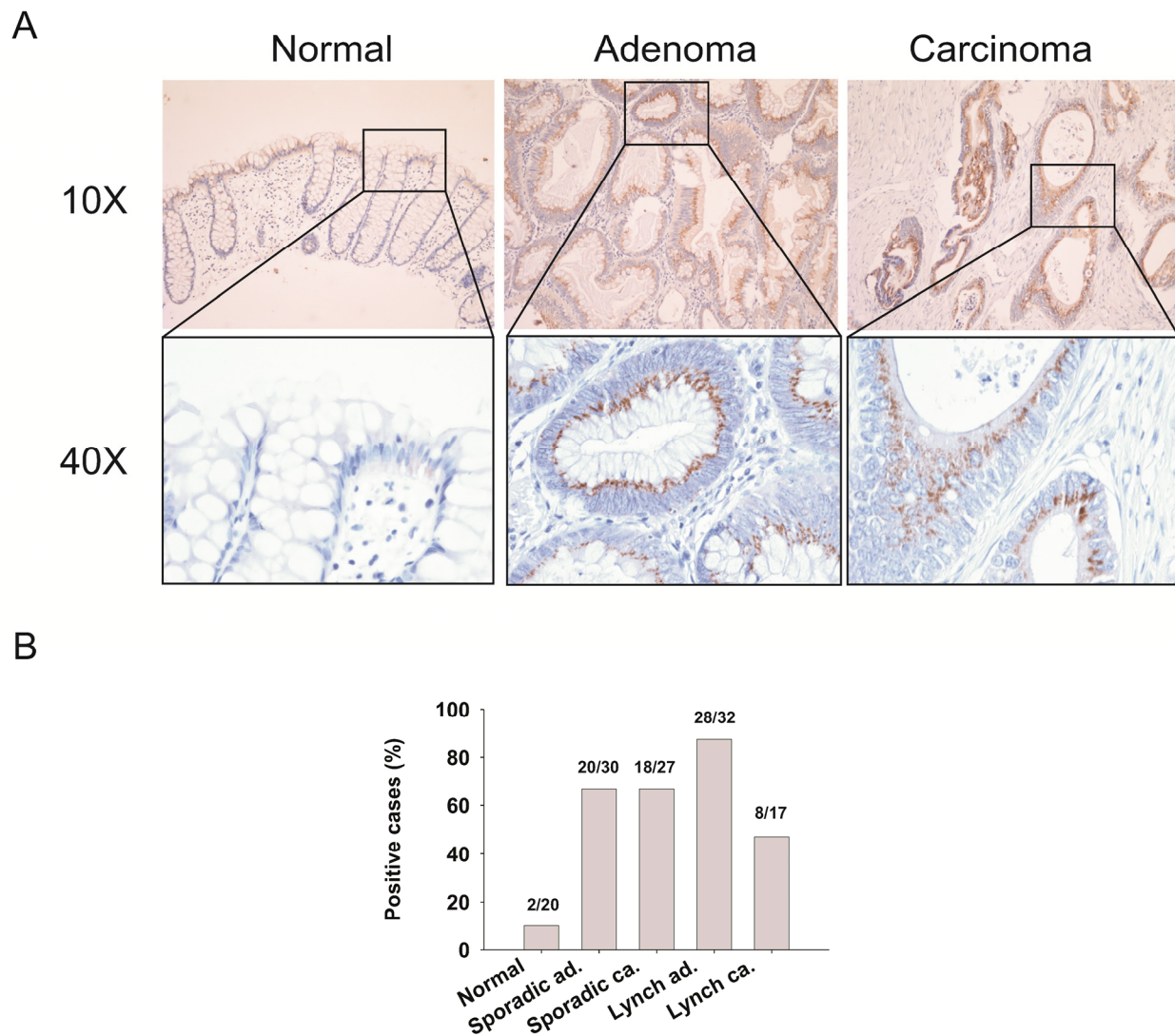


Figure 5: Mcl-1 expression is increased during colorectal carcinogenesis. **A:** Representative examples of Mcl-1 expression in normal colonic mucosa with negative perinuclear staining but moderate diffuse staining, in colorectal adenoma and in carcinoma, both with positive perinuclear staining, shown at the 10 and 40 times magnification. **B:** Expression of Mcl-1 in % positive cases in normal colonic mucosa, sporadic adenomas, sporadic carcinomas, Lynch syndrome adenomas and Lynch syndrome carcinomas.

Discussion

RhTRAIL, in combination with either chemotherapy or targeted drugs, is highly effective in inducing apoptosis in colon cancer cells (34). We now demonstrate that combining the drugs aspirin and sorafenib, at clinically relevant concentrations, strongly inhibits colon cancer cell viability. Next, we show that at the same concentrations, the combination of aspirin and sorafenib is much more effective at enhancing rhTRAIL-induced apoptosis than either drug alone. The mechanism of this rhTRAIL sensitization by aspirin and sorafenib was identified, namely FLIP and Mcl-1 protein downregulation. These two proteins were found to be highly expressed in human colon cancer samples, confirming the relevance of our findings. The results of this study regarding the strong potentiating effect of low doses of aspirin and sorafenib on rhTRAIL sensitivity in colon cancer cells, including rhTRAIL-resistant cells, should therefore stimulate further clinical development of this targeted drug combination.

TRAIL resistance is often due to high levels of anti-apoptotic proteins; this can be reversed with inhibition of their expression. We find that the increase in rhTRAIL sensitivity following aspirin and sorafenib cotreatment was caused by a time-dependent downregulation in levels of the. FLIP is a widely accepted inhibitor of TRAIL sensitivity at the DISC (26) and has also been shown to induce resistance to chemotherapy in colon cancer cells (35,36). FLIP expression is increased in colorectal cancer, supporting the potential of combining FLIP-targeting strategies with TRAIL receptor agonists (21). The influence of Mcl-1 expression in association with colon carcinogenesis is less evident. By comparing human adenomatous polyps and primary colorectal adenocarcinomas to their adjacent normal mucosa, Krajewska *et al.* found stronger Mcl-1 immunointensity in 29% of adenomas but lower immunointensity in 67% of primary cancers (37). Comparing Mcl-1 expression in four colon carcinomas with their surrounding epithelium, Schulze-Bergkamen *et al.* reported that Mcl-1 expression was slightly higher in colon cancer (38). Using the same patient material as in our study on FLIP expression, we show here that Mcl-1 expression is increased during colorectal carcinogenesis. As suggested for Bcl-2 (39), Mcl-1 might provide a survival advantage to colon adenoma and carcinoma cells compared to healthy cells due to its anti-apoptotic properties. In contrast to previous studies, clearly higher expression of Mcl-1 was seen in adenomas but also in carcinomas compared to normal colonic tissue. Abnormality of the colonic

mucosa up to several centimeters away from the edge of a tumors has been suggested previously (39), and might explain the discrepancies with earlier studies on Mcl-1. Altogether, our results support that Mcl-1 protein expression increases early during colorectal carcinogenesis and is largely conserved in cancer.

We show that the combination of aspirin and sorafenib increases rhTRAIL-sensitivity in the Bax-deficient Lovo cell line. This is of particular importance since Bax is frequently lost in colon cancer (up to 50% in Lynch syndrome patients) and plays an important role in acquisition of rhTRAIL resistance *in vitro* and *in vivo* (40). In contrast, the NSAID sulindac sulfide induced TRAIL sensitization via a Bax-dependent mechanism by decreasing Bcl-x_L expression in colorectal cancer cells (41). Since Bak is less frequently lost in colon cancer than Bax, it might be interesting to exploit the potential of Bak to activate the mitochondrial pathway. Notably, we found that the combination of aspirin and sorafenib reduced expression of the well-known Bak inhibitor Mcl-1 (27) and sensitized colon cancer cells to rhTRAIL-induced apoptosis in a Bak-dependent manner. No specific agent targeting Mcl-1 has been developed yet. Although Obatoclax targets the Bcl-2 family proteins including Mcl-1, it was found to induce apoptosis in cells lacking Bax and Bak expression as well, suggesting mitochondria-independent effects on apoptosis (42). Wei *et al.* previously showed that simultaneous knock-out of Bax and Bak was necessary for impairing Bid-mediated apoptosis, suggesting that these two proteins were redundant in function (43). Based notably on experiments performed in the Bax-proficient and Bax-deficient HCT116 cell model, the current paradigm has been that Bax but not Bak is critical for the TRAIL-induced activation of the mitochondrial pathway in cancer cells (40,44). Our Bak siRNA experiments partially challenge this assumption since the type II cell line Lovo is initially moderately sensitive to rhTRAIL, whereas it only expresses Bak. Furthermore, Bak siRNA completely blocks rhTRAIL-induced apoptosis in HCT116 and Lovo, further suggesting the importance of this protein. Chemotherapeutic drugs modify the balance between pro and anti-apoptotic Bcl-2 family members, often with preferential activation of Bax or Bak (45-47). A better knowledge on the mechanisms and proteins regulating Bid-induced activation of Bax and Bak appears needed to improve mitochondrial activation following TRAIL treatment.

It is noteworthy that little to no apoptosis was induced by aspirin and sorafenib cotreatment alone despite downregulation of FLIP and Mcl-1. Rather, aspirin and

sorafenib cotreatment had a strong potentiating effect on growth inhibition. Previous reports have shown that both aspirin and sorafenib inhibit expression of cell cycle-related proteins associated with G₁-S progression such as cyclin D1 and ppRb (8,48,49). Sorafenib prevented cell survival in all colon cancer cell lines tested *in vitro*. The effects of sorafenib alone were independent of the B-Raf status, as previously observed in melanoma cell lines by Plataras *et al.* (8). We now show that aspirin can potentiate the effects of sorafenib in colon cancer cells. An increase in the proportion of cells in G₁ and a decrease in number of cells in S phase were observed, suggesting that the combination delays cell cycle progression. Accordingly, a decrease in expression of cyclin D1 and ppRb was also seen. We cannot rule out that the combination of aspirin and sorafenib might also inhibit different phases of the cell cycle depending on the cell line used. Sorafenib has been shown to exert cell line-dependent effects on cell cycle progression (8). Since the anti-proliferative effects of the combination of aspirin with sorafenib were seen both in wild type and mutant Raf cells, response to the combination seems independent of the mutational status of the Raf/MEK/ERK pathway.

In conclusion, aspirin and sorafenib potentiate each other in preventing colon cancer cell proliferation by inhibiting cell cycle progression. Combining these two drugs at low concentrations also markedly increases colon cancer cell sensitivity to rhTRAIL-induced apoptosis. This is due to a decrease in levels of the proteins FLIP and Mcl-1, both of which we found to be over-expressed in human colon cancer specimens. Therefore, our results provide a strong rationale to explore the clinical potential of aspirin, sorafenib and TRAIL receptor agonists in the treatment of colon cancer.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T and Thun M. Cancer Statistics, 2008. *CA Cancer J Clin* 2008; 58: 71-96.
2. Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresalier R, McKeown-Eyssen G, Summers RW, Rothstein R, Burke CA, Snover DC, Church TR, Allen JI, Beach M, Beck GJ, Bond JH, Byers T, Greenberg ER, Mandel JS, Marcon N, Mott LA, Pearson L, Saibil F and van Stolk RU. A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med* 2003; 348: 891-899.
3. Jalving M, Koornstra JJ, de Jong S, de Vries EGE and Kleibeuker JH. Review article: the potential of combinational regimen with non-steroidal anti-inflammatory drugs in the chemoprevention of colorectal cancer. *Aliment Pharmacol Ther* 2005; 21: 321-339.
4. El Rayes BF, Zalupski MM, Manza SG, Rusin B, Ferris AM, Vaishampayan U, Heilbrun LK, Venkatramanamoorthy R, Shields AF and Philip PA. Phase-II study of dose attenuated schedule of irinotecan, capecitabine, and celecoxib in advanced colorectal cancer. *Cancer Chemother Pharmacol* 2008; 61: 283-289.
5. Maiello E, Giuliani F, Gebbia V, Di Renzo N, Pezzella G, Romito S, Mallamaci R, Lopez M and Colucci G. FOLFIRI with or without celecoxib in advanced colorectal cancer: a randomized phase II study of the Gruppo Oncologico dell'Italia Meridionale (GOIM). *Ann Oncol* 2006; 17 Suppl 7: vii55-vii59.
6. de Groot DJ, de Vries EGE, Groen HJ and de Jong S. Non-steroidal anti-inflammatory drugs to potentiate chemotherapy effects: from lab to clinic. *Crit Rev Oncol Hematol* 2007; 61: 52-69.
7. Wilhelm S, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujath J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnane L, Lynch M, Auclair D, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post L, Bollag G and Trail P. BAY 43-9006 Exhibits Broad Spectrum Oral Antitumor Activity and Targets the RAF/MEK/ERK Pathway and Receptor Tyrosine Kinases Involved in Tumor Progression and Angiogenesis. *Cancer Res* 2004; 64: 7099-7109.
8. Plastaras JP, Kim SH, Liu YY, Dicker DT, Dorsey JF, McDonough J, Cerniglia G, Rajendran RR, Gupta A, Rustgi AK, Diehl JA, Smith CD, Flaherty KT and El Deiry WS. Cell cycle dependent and schedule-dependent antitumor effects of sorafenib combined with radiation. *Cancer Res* 2007; 67: 9443-9454.
9. Yu C, Bruzek LM, Meng XW, Gores GJ, Carter CA, Kaufmann SH and Adjei AA. The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. *Oncogene* 2005; 24: 6861-6869.
10. Perugini RA, McDade TP, Vittimberga FJ, Jr., Duffy AJ and Callery MP. Sodium salicylate inhibits proliferation and induces G1 cell cycle arrest in human pancreatic cancer cell lines. *J Gastrointest Surg* 2000; 4: 24-32.
11. Iglesias-Serret D, Pique M, Gil J, Pons G and Lopez JM. Transcriptional and translational control of Mcl-1 during apoptosis. *Arch Biochem Biophys* 2003; 417: 141-152.

12. Johnstone RW, Frew AJ and Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer* 2008; 8: 782-798.
13. Koornstra JJ, Kleibeuker JH, van Geelen CMM, Rijcken FE, Hollema H, de Vries EGE and de Jong S. Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonic mucosa, adenomas, and carcinomas. *J Pathol* 2003; 200: 327-335.
14. Hague A, Hicks DJ, Hasan F, Smartt H, Cohen GM, Paraskeva C and MacFarlane M. Increased sensitivity to TRAIL-induced apoptosis occurs during the adenoma to carcinoma transition of colorectal carcinogenesis. *Br J Cancer* 2005; 92: 736-742.
15. Jalving M, de Jong S, Koornstra JJ, Boersma-van Ek W, Zwart N, Wesseling J, de Vries EGE and Kleibeuker JH. TRAIL induces apoptosis in human colorectal adenoma cell lines and human colorectal adenomas. *Clin Cancer Res* 2006; 12: 4350-4356.
16. Oldenhuis CNAM, Stegehuis JH, Walenkamp AME, de Jong S and de Vries EGE. Targeting TRAIL death receptors. *Curr Opin Pharmacol* 2008; 8: 433-439.
17. Soria J, Smit E, Khayat D, Besse B, Yang X, Hsu C, Reese D, Wiezorek J and Blackhall F. Phase 1b Study of Dulanermin (recombinant human Apo2L/TRAIL) in Combination With Paclitaxel, Carboplatin, and Bevacizumab in Patients With Advanced Non-Squamous Non-Small-Cell Lung Cancer. *J Clin Oncol* 2010; 28: 1527-1533.
18. van Geelen CMM, de Vries EGE, Le TKP, van Weeghel RP and de Jong S. Differential modulation of the TRAIL receptors and the CD95 receptor in colon carcinoma cell lines. *Br J Cancer* 2003; 89: 363-373.
19. Wirth T, Kuhnel F, Fleischmann-Mundt B, Woller N, Djojotubroto M, Rudolph K, Manns M, Zender L and Kubicka S. Telomerase-Dependent Virotherapy Overcomes Resistance of Hepatocellular Carcinomas against Chemotherapy and Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand by Elimination of Mcl-1. *Cancer Res* 2005; 65: 7393-7402.
20. Shim E, Lee YS, Kim HY and Jeoung D. Down-regulation of c-FLIP increases reactive oxygen species, induces phosphorylation of serine/threonine kinase Akt, and impairs motility of cancer cells. *Biotechnol Lett* 2007; 29: 141-147.
21. Heijink DM, Kleibeuker JH, Jalving M, Boersma-van Ek W, Koornstra JJ, Wesseling J and de Jong S. Independent induction of caspase-8 and cFLIP expression during colorectal carcinogenesis in sporadic and HNPCC adenomas and carcinomas. *Cell Oncol* 2007; 29: 409-419.
22. Krajewski S, Bodrug S, Krajewska M, Shabaik A, Gascoyne R, Berean K and Reed JC. Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death in vivo. *Am J Pathol* 1995; 146: 1309-1319.
23. Clark JW, Eder JP, Ryan D, Lathia C and Lenz HJ. Safety and pharmacokinetics of the dual action Raf kinase and vascular endothelial growth factor receptor inhibitor, BAY 43-9006, in patients with advanced, refractory solid tumors. *Clin Cancer Res* 2005; 11: 5472-5480.

24. Calleros L, Sanchez-Hernandez I, Baquero P, Toro M and Chiloeches A. Oncogenic Ras, but not V600EB-RAF, protects from cholesterol depletion-induced apoptosis through the PI3K/AKT pathway in colorectal cancer cells. *Carcinogenesis* 2009; 30: 1670-1677.
25. Hao H, Muniz-Medina V, Mehta H, Thomas N, Khazak V, Der C and Shields J. Context-dependent roles of mutant B-Raf signaling in melanoma and colorectal carcinoma cell growth. *Mol Cancer Ther* 2007; 6: 2220-2229.
26. Krueger A, Baumann S, Krammer PH and Kirchhoff S. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol Cell Biol* 2001; 21: 8247-8254.
27. Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM and Huang DC. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* 2005; 19: 1294-1305.
28. Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC and Perucho M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997; 275: 967-969.
29. Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B and Martinou JC. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* 1999; 144: 891-901.
30. Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB and Korsmeyer SJ. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev* 2000; 14: 2060-2071.
31. Barnhart BC, Alappat EC and Peter ME. The CD95 type I/type II model. *Semin Immunol* 2003; 15: 185-193.
32. Wilson TR, McEwan M, McLaughlin K, Le Clorennec C, Allen WL, Fennell DA, Johnston PG and Longley DB. Combined inhibition of FLIP and XIAP induces Bax-independent apoptosis in type II colorectal cancer cells. *Oncogene* 2009; 28: 63-72.
33. Hector S and Prehn J. Apoptosis signaling proteins as prognostic biomarkers in colorectal cancer: A review. *BBA-Rev Cancer* 2009; 1795: 117-129.
34. van Geelen CMM, de Vries EGE and de Jong S. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist Updat* 2004; 7: 345-358.
35. Longley DB, Wilson TR, McEwan M, Allen WL, McDermott U, Galligan L and Johnston PG. c-FLIP inhibits chemotherapy-induced colorectal cancer cell death. *Oncogene* 2006; 25: 838-848.
36. Wilson TR, McLaughlin KM, McEwan M, Sakai H, Rogers KM, Redmond KM, Johnston PG and Longley DB. c-FLIP: a key regulator of colorectal cancer cell death. *Cancer Res* 2007; 67: 5754-5762.
37. Krajewska M, Moss SF, Krajewski S, Song K, Holt PR and Reed JC. Elevated expression of Bcl-X and reduced Bak in primary colorectal adenocarcinomas. *Cancer Res* 1996; 56: 2422-2427.

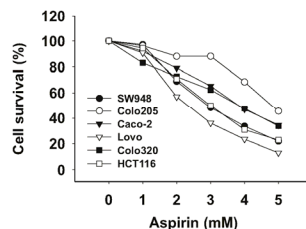
38. Schulze-Bergkamen H, Ehrenberg R, Hickmann L, Vick B, Urbanik T, Schimanski CC, Berger MR, Schad A, Weber A, Heeger S, Galle PR and Moehler M. Bcl-x(L) and Myeloid cell leukaemia-1 contribute to apoptosis resistance of colorectal cancer cells. *World J Gastroenterol* 2008; 14: 3829-3840.
39. Koornstra JJ, de Jong S, Hollema H, de Vries EGE and Kleibeuker JH. Changes in apoptosis during the development of colorectal cancer: a systematic review of the literature. *Crit Rev Oncol Hematol* 2003; 45: 37-53.
40. LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D and Ashkenazi A. Tumor-cell resistance to death receptor--induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nat Med* 2002; 8: 274-281.
41. Ravi R and Bedi A. Requirement of BAX for TRAIL/Apo2L-induced apoptosis of colorectal cancers: synergism with sulindac-mediated inhibition of Bcl-x(L). *Cancer Res* 2002; 62: 1583-1587.
42. Mott J, Bronk S, Mesa R, Kaufmann S and Gores G. BH3-only protein mimetic obatoclax sensitizes cholangiocarcinoma cells to Apo2L/TRAIL-induced apoptosis. *Mol Cancer Ther* 2008; 7: 2339-2347.
43. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB and Korsmeyer SJ. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001; 292: 727-730.
44. Deng Y, Lin Y and Wu X. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. *Genes Dev* 2002; 16: 33-45.
45. von Haefen C, Gillissen B, Hemmati PG, Wendt J, Guner D, Mrozek A, Belka C, Dorken B and Daniel PT. Multidomain Bcl-2 homolog Bax but not Bak mediates synergistic induction of apoptosis by TRAIL and 5-FU through the mitochondrial apoptosis pathway. *Oncogene* 2004; 23: 8320-8332.
46. Wang S and El Deiry WS. Requirement of p53 targets in chemosensitization of colonic carcinoma to death ligand therapy. *Proc Natl Acad Sci U S A* 2003; 100: 15095-15100.
47. Wang SH, Mezosi E, Wolf JM, Cao Z, Utsugi S, Gauger PG, Doherty GM and Baker JR, Jr. IFN γ sensitization to TRAIL-induced apoptosis in human thyroid carcinoma cells by upregulating Bak expression. *Oncogene* 2004; 23: 928-935.
48. Sherr C and McCormick F. The RB and p53 pathways in cancer. *Cancer Cell* 2002; 2: 103-112.
49. Takada Y, Bhardwaj A, Potdar P and Aggarwal B. Nonsteroidal anti-inflammatory agents differ in their ability to suppress NF-kappaB activation, inhibition of expression of cyclooxygenase-2 and cyclin D1, and abrogation of tumor cell proliferation. *Oncogene* 2004; 23: 9247-9258.

Supplementary Figures

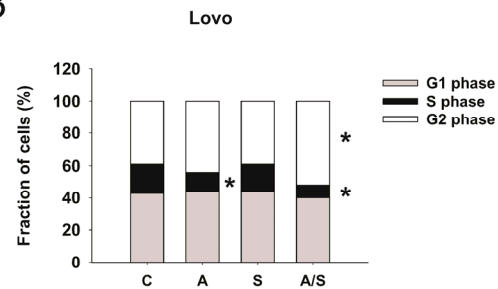
S1

	GI ₅₀ (μM)
SW948	2,4
Colo205	4,2
Caco-2	4,7
Lovo	3,8
Colo320	4,3
HCT116	1,7

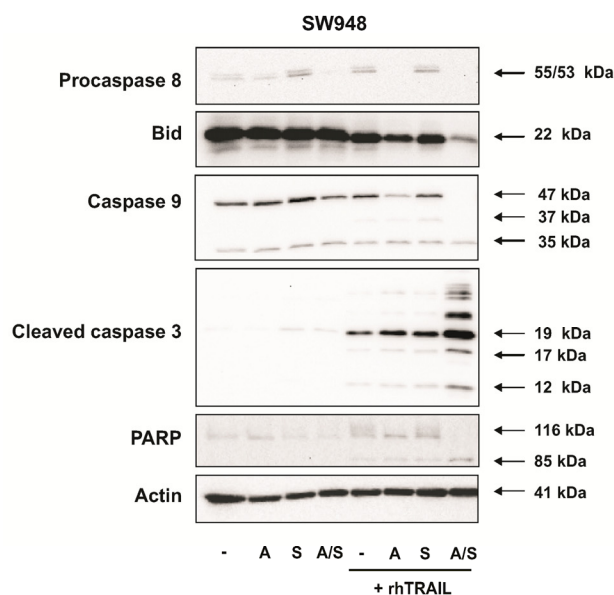
S2



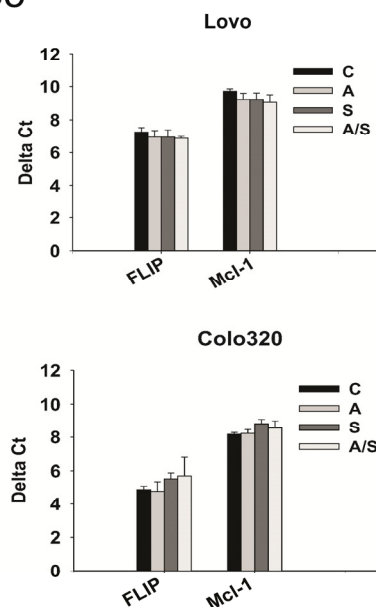
S3



S4



S5



S1: GI₅₀ of various colon cancer cell lines for sorafenib.

S2: Sensitivity of colon cancer cell lines to aspirin. Cells were treated for 96 h with the indicated drug concentrations before cell survival determination.

S3: Effects of an incubation with aspirin (A) (2 mM), sorafenib (S) (2 μM) or the combination (A/S) on cell cycle distribution in Lovo. Cells were treated as described in Figure 1C before analysis. Values are mean of at least 3 independent experiments.

S4: Western-blot analysis showing activation of caspases, Bid and PARP in SW948 cells pre-treated with aspirin (2 mM) and/or sorafenib (2 μM) for 24 h before 17 h-treatment with rhTRAIL (10 ng/mL).

S5: The combination of sorafenib and aspirin does not downregulate FLIP and Mcl-1 mRNA levels. Effects of a 41 h-incubation with aspirin, sorafenib or both drugs in combination on FLIP and Mcl-1 mRNA levels in Colo320 and Lovo cells.

Chapter 7

Summary of results, discussion and future perspectives

Summary of results

Colorectal cancer is the second cause of cancer-related death in western countries. Although therapeutic progress has been made, intrinsic and/or acquired drug resistance remains a problem, which is probably caused by disruption of the apoptosis pathway in colon cancer cells. Both anti-tumor and anti-metastatic roles have been suggested for the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptotic pathway *in vivo*. This makes the TRAIL apoptotic pathway a therapeutic target. Novel agents - among them, recombinant human (rh)TRAIL and agonistic antibodies targeting either TRAIL receptor 1 (TRAIL-R1) or TRAIL-R2 - have been designed to stimulate the extrinsic pathway of apoptosis in cancer cells. Combination therapies making use of these agents are currently being evaluated for their ability to re-establish apoptosis in chemotherapy-resistant cells. This thesis investigated various aspects of the TRAIL apoptotic pathway, with the goal to improve the molecular targeting of this pathway in colon cancer.

To delineate new strategies leading to higher sensitivity to TRAIL death receptor-mediated apoptosis, we first reviewed current knowledge on the initiating step of the pathway, the so-called death-inducing signaling complex (DISC) formation (**chapter 2**). DISC assembly is triggered in cancer cells via binding of TRAIL to its two proapoptotic receptors, TRAIL-R1 and TRAIL-R2. Primary components of the DISC are trimerized TRAIL-R1/-R2, FADD, caspase 8 and caspase 10. The anti-apoptotic protein FLIP can also be recruited to the DISC to replace caspase 8, thereby forming an inactive complex. Caspase 8/10 processing at the DISC triggers the caspase cascade, which eventually leads to apoptotic cell death. Besides TRAIL, TRAIL-R1- and TRAIL-R2-selective variants of TRAIL and agonistic antibodies have been designed. These ligands are of interest as anti-cancer agents, since they selectively kill tumor cells. To increase tumor sensitivity to TRAIL death receptor-mediated apoptosis and to overcome drug resistance, TRAIL receptor ligands have already been combined with various therapies in preclinical models. In **chapter 2**, we discuss factors influencing the initial steps of TRAIL apoptosis pathway signaling, focusing on mechanisms modulating DISC assembly and caspase activation at the DISC. These insights can direct rational design of drug combinations with TRAIL receptor ligands to maximize DISC signaling.

One way to identify combinations that increase TRAIL-induced apoptosis has been the development of cancer cell line models for TRAIL resistance, often in mismatch repair-deficient colon cancer cells. These cells are prone to mutations in genes containing tandem repeats, including the gene encoding for the pro-apoptotic protein Bax, but they only represent a small fraction of colorectal cancers (15-20%). In **chapter 3**, we investigated the mechanism underlying TRAIL resistance acquisition in a mismatch repair-proficient colon carcinoma cell line. The TRAIL-resistant cell line SW948-TR was established from the TRAIL-sensitive cell line SW948 by continuous exposure to rhTRAIL, and exhibited a 140-fold lower sensitivity to rhTRAIL in a cell viability assay. Resistance was stable for over a year in the absence of rhTRAIL. Both cell lines had similar TRAIL receptor cell membrane expression levels. Treatment with the protein synthesis inhibitor cycloheximide sensitized SW948-TR to rhTRAIL-induced apoptosis, indicating that the functionality of the TRAIL receptors was maintained. In SW948-TR, procaspase 8 protein levels but not mRNA levels were notably lower than in SW948. Downregulation of c-FLIP with short interfering RNA (siRNA) sensitized SW948-TR cells to rhTRAIL, while caspase 8 siRNA decreased rhTRAIL sensitivity in SW948, highlighting the importance of the caspase 8/FLIP ratio. Proteasome inhibition with MG-132 did not restore basic procaspase 8 levels but stabilized cleaved caspase 8 in rhTRAIL-treated SW948-TR cells. Altogether, our results suggest that colon cancer cells may acquire rhTRAIL resistance by primarily reducing the basal procaspase 8/FLIP ratio and by increasing active caspase 8 degradation after rhTRAIL treatment. Proteasome inhibitors may effectively overcome acquired rhTRAIL resistance in mismatch repair-proficient colon cancer cells.

Several agents have been designed to target the TRAIL receptors, including TRAIL receptor-specific ligands. Because these various ligands might have different properties, we investigated in **chapter 4** the contribution of TRAIL-R1 and of TRAIL-R2 to rhTRAIL-sensitivity and rhTRAIL-resistance in the SW948 and SW948-TR cell line model. We also assessed whether anti-TRAIL-R1 or anti-TRAIL-R2 agonistic antibodies could circumvent rhTRAIL resistance. SW948 cells were sensitive to all three of the TRAIL receptor-targeting agents tested, although the anti-TRAIL-R2 antibody induced only weak caspase 8 cleavage and low apoptosis levels. Nonetheless, anti-TRAIL-R1 and anti-TRAIL-R2 antibodies induced equivalent DISC formation as well as equivalent caspase 8 cleavage at the level of their individual receptors. This indicates

that further caspase 8 processing is impaired upon stimulation of TRAIL-R2. SW948-TR cells were resistant to all TRAIL receptor-targeting agents due to their lower caspase 8 expression levels. Caspase 8 levels could be restored by MG-132 or interferon- γ (IFN- γ) pretreatment, and sensitivity to rhTRAIL and the anti-TRAIL-R1 antibody was increased. Surprisingly, MG-132 also enhanced TRAIL-R2-mediated apoptosis. These results highlight a critical difference between TRAIL-R1 and TRAIL-R2 apoptotic signaling modulation, with possible implications for future combinatorial regimens.

The activity of the insulin-like growth factor 1 (IGF-1) survival pathway is often increased in cancer, influencing both cell proliferation and apoptosis. In **chapter 5**, we hypothesized that inhibiting the IGF-1 receptor (IGF-1R) could increase TRAIL death receptor-mediated apoptosis in colon cancer cells. NVP-AEW541, a small molecular weight tyrosine kinase inhibitor of the IGF-1R, surprisingly decreased sensitivity to rhTRAIL and to an anti-TRAIL-R1 antibody; sensitivity to an anti-TRAIL-R2 antibody was increased. NVP-AEW541 did inhibit IGF-1-induced activation of the phosphatidylinositol 3-kinase (PI3K) pathway. The effects of the PI3K inhibitor LY294002 on TRAIL-induced apoptosis were similar to those of NVP-AEW541, further supporting a role for IGF-1R-mediated activation of PI3K. We show that PI3K inhibition enhances TRAIL-R2-mediated caspase 8 processing but at the same time lowers TRAIL-R1 membrane expression and TRAIL-R1-mediated caspase 8 processing. Inhibition of PI3K reduced rhTRAIL sensitivity, an effect which was independent of the cell line's initial preference for either TRAIL-R1- or TRAIL-R2-mediated apoptosis signaling. These results suggest that individual effects on TRAIL-R1 and TRAIL-R2 apoptosis signaling should be taken into consideration when combining TRAIL receptor ligands with PI3K inhibition.

The multikinase inhibitor sorafenib is highly effective against certain types of cancer in the clinic and prevents colon cancer cell proliferation *in vitro*. Non-steroidal anti-inflammatory drugs such as acetylsalicylic acid (aspirin) have shown activity against colon cancer cells. The aims of **chapter 6** were to determine whether the combination of aspirin with sorafenib has enhanced anti-proliferative effects and increases rhTRAIL-induced apoptosis in the human SW948, Lovo, Colo205, Colo320, Caco-2, and HCT116 colon cancer cell lines. In four cell lines, aspirin strongly stimulated the anti-proliferative effects of sorafenib (~ four-fold enhancement) by inducing cell cycle arrest. Furthermore, combining low doses of aspirin (≤ 2.5 mM) and sorafenib (\leq

2.5 μ M) greatly sensitized TRAIL-sensitive and TRAIL-resistant colon cancer cells to rhTRAIL. The increase in rhTRAIL sensitivity was caused by a reduction in basal levels of the anti-apoptotic proteins FLIP and Mcl-1 following aspirin and sorafenib cotreatment, as confirmed with small interfering RNA. Next, the clinical relevance of targeting FLIP and Mcl-1 in colon cancer was examined. Using immunohistochemistry, we found that Mcl-1 expression was significantly increased in colon adenoma and carcinoma patient material compared to healthy colonic epithelium, similar to the enhanced FLIP expression we recently observed in colon cancer. These results underscore the potential of combining low doses of aspirin with sorafenib to inhibit proliferation and target the anti-apoptotic proteins FLIP and Mcl-1. Our findings also support further pre(clinical) investigation of this combination with TRAIL receptor-targeting agents in colon cancer.

Discussion and future perspectives

Resistance of colorectal cancer cells to standard 5-fluorouracil (FU)-based chemotherapy can be attributed in part to a failure of cancer cells to go into apoptosis. So far, most chemotherapeutic agents used to treat colorectal cancer have targeted the intrinsic apoptosis pathway (1). Agents activating the TRAIL apoptotic pathway offer a novel strategy to complement current chemotherapeutic regimens by stimulating the extrinsic apoptosis pathway, without toxicity toward normal cells. Since the discovery of TRAIL receptors in 1997, much emphasis has been put on defining the optimal drug combination(s) to increase cancer cell sensitivity to TRAIL, and on identifying the key proteins regulating the TRAIL apoptotic pathway.

Properties of TRAIL-R1 and TRAIL-R2

Although the two pro-apoptotic TRAIL death receptors were initially described as functionally homologous, a significant body of work suggests that they also possess some distinct properties. This neglected aspect might be particularly important in view of the wide range of TRAIL receptor agonists currently in development (2). To summarize, TRAIL-R1 expression is principally regulated by stress-activated pathways such as c-Jun, while the tumor suppressor p53 plays a crucial role in regulating TRAIL-R2 expression (3). In some cancer cell types, such as chronic lymphoid leukaemia,

TRAIL mostly signals apoptosis via TRAIL-R1. Not surprisingly, loss of TRAIL-R1 expression is more frequent than loss of TRAIL-R2 expression in this type of tumor (2). Moreover, the two pro-apoptotic receptors also have distinct prognostic value in various cancer types (4). Upon ligand stimulation, specific proteins can be recruited to either TRAIL-R1- or TRAIL-R2-associated DISC complexes where they modulate caspase 8 activation and/or stimulate various downstream survival pathways (2,5). These properties might contribute to the various efficacies of the different TRAIL receptor ligands, alone or in combination with cytotoxic drugs.

TRAIL receptor ligand-specific properties

In chapters 4 and 5, we showed that antibodies specific for TRAIL-R1 or TRAIL-R2 might not always achieve the levels of cell death reached by the dual TRAIL-R1/-R2-agonist rhTRAIL. As we highlight in chapter 4, in the isogenic SW948/SW948-TR colon cancer cell line model, the anti-TRAIL-R2 antibody was less potent than rhTRAIL or the anti-TRAIL-R1 antibody. Our results further show that these inequalities between ligands were conserved when sensitivity to apoptosis was increased with the therapeutic agent IFN- γ . The proteasome inhibitor MG-132 could, however, improve TRAIL-R2-mediated apoptosis to the levels of TRAIL-R1-mediated apoptosis. Based on these results, rhTRAIL or simultaneous treatment with TRAIL-R1 and TRAIL-R2 agonists might seem to be the best workaround options since potential TRAIL-R1- and TRAIL-R2-specific drug effects could be bypassed. However, our data in chapter 5 partially challenge this assumption.

In chapter 5, we showed that agents targeting the IGF-1R/PI3K survival pathway, in this case NVP-AEW541 or LY294002, decreased colon cancer cell sensitivity to rhTRAIL and to anti-TRAIL-R1 antibodies, while sensitivity to anti-TRAIL-R2 antibodies was increased. This was seen both in the SW948/SW948-TR cell line model and in the Colo205 cell line. This implies that for a given treatment option, a particular TRAIL receptor agonist may have to be used to avoid a possible lack of potentiating effects or, even more dramatically, potential antagonistic effects. Unlike TRAIL-R1 or TRAIL-R2-specific agonists, rhTRAIL can recruit TRAIL receptor heterotrimers; their ability to transduce apoptosis, compared to homotrimers, remains to be further investigated. In addition to their effects on the apoptotic cascade, the propensity of the various TRAIL receptor ligands and/or TRAIL death receptor to co-activate pro-

survival pathways largely remains to be investigated (5). Altogether, *in vitro* results on the efficacy of the various ligands should be expanded to larger cell line panels and substantiated by human tumor xenograft animal model data. This would allow evaluating the translation of these TRAIL receptor agonist-specific properties into *in vivo* models.

Very few studies have compared the efficiency of TRAIL-R1- and TRAIL-R2-agonists *in vitro* to their efficiency *in vivo* in preclinical models. The results obtained so far suggest that, to some extent, the response to anti-TRAIL-R1 and anti-TRAIL-R2 antibodies alone observed *in vitro* hold some predictive value on response in xenograft models (6). However, *in vitro* data on the combinations of the anti-TRAIL-R1 and anti-TRAIL-R2 antibodies with ionizing radiation did not concur with the gain in response seen *in vivo* in xenograft models. Clinical data comparing the efficacy of two (or more) TRAIL receptor ligands in combination with the same anti-cancer therapeutic regimen are also sparse. Our *in vitro* data support the idea that standard anti-cancer treatments might have TRAIL receptor ligand-specific properties, which should be taken into consideration when combining these treatments with a particular ligand.

Determining the functionality of the TRAIL-R1- and TRAIL-R2-apoptotic signaling pathways in a given tumor could help to define the optimal TRAIL receptor agonist to be used. If possible, functionality should be based on *ex vivo* response to the TRAIL receptor agonist(s), alone or upon combination with the standard treatment option(s). Cultures of precision-cut human tumor tissue slices or human xenotransplants in mice could be used to determine optimal drug-response. Preclinical data, including results obtained in isogenic colon cancer cell lines, suggest that primary tumors are more sensitive to TRAIL than their metastases (7,8). Therefore, it will be important to determine whether the response of primary tumor material to the TRAIL receptor ligands, either alone or in combination with cytotoxic drugs, can also predict response of the metastases to these combinations. Tumor specific- and, by extension, patient tailored-TRAIL receptor agonist combination therapies may be very effective, but necessitate a major leap in screening modalities. Until advanced screening strategies are implemented, it may also be useful to characterize the most effective TRAIL receptor ligand(s) for each cancer type.

Critical role of the DISC and importance of (a high) caspase 8/FLIP ratio for TRAIL sensitivity

A large amount of data has been accumulated on how to further sensitize cells to TRAIL death receptor-mediated apoptosis by stimulating the intrinsic apoptosis pathway. The review presented in chapter 2 argues that TRAIL sensitivity should already be improved at the DISC. Optimal caspase 8 activation at the DISC signifies better activation of Bid upstream of the mitochondria and better ability to directly activate executioner caspases such as caspase 3. Further knowledge on the mechanisms that determine the efficiency of caspase 8 cleavage is therefore needed. The ability of the various TRAIL receptor ligands to initiate efficient DISC formation, the influence of TRAIL-R1 or TRAIL-R2 mutations on caspase 8 cleavage and the intracellular mechanisms modulating DISC activity should be carefully examined (2).

Resistance to TRAIL can be intrinsic, but it can also develop in a subset of cells after repeated exposure to TRAIL. We show that procaspase 8 downregulation and active caspase 8 degradation play a role in MMR-proficient cells with long-term acquired rhTRAIL resistance. One of the mechanisms by which anti-cancer drugs increase TRAIL sensitivity is by enhancing the expression of pro-apoptotic proteins at the DISC. We show in chapter 3 and 4 that both proteasome inhibitors and IFN- γ increase caspase 8 levels, thereby reversing rhTRAIL-resistance in SW948-TR cells. Future studies should therefore aim at defining optimal TRAIL-based therapies for increasing caspase 8 activation. In a broader perspective, our results demonstrate the importance of the caspase 8/FLIP ratio for TRAIL sensitivity. Drugs that decrease FLIP protein levels can enhance response to chemotherapy and TRAIL-induced apoptosis in colorectal cancer cells (9,10). FLIP-specific targeted therapeutic agents are currently awaited.

The parental TRAIL-sensitive cell line used in chapter 3 is classified as a type I cell line, characterized by potent caspase 8 cleavage at the DISC and independence from the mitochondria for TRAIL-induced apoptosis, as determined *in vitro*. Alteration in the TRAIL apoptotic pathway at the level of the DISC itself, for instance through caspase 8 downregulation, seems expected in a type I cell line that has acquired resistance to TRAIL. However, many cancer cells are classified type II and the mitochondrial amplification loop is critical for apoptosis in these cells. It would be of interest to test whether the acquisition of TRAIL-resistance in type II cells also correlates with changes

in DISC component levels, or promotes alterations in proteins involved in regulation of the mitochondrial apoptotic pathway.

In view of the broad range of mechanisms leading to acquired TRAIL resistance, studies on the selective pressure induced by prolonged TRAIL treatment should preferably be performed in a broad range of colon cancer cell lines. This would allow assessing the relevance of the resistance mechanisms described and permit characterizing agents that re-sensitize the widest panel of cell lines.

Additional targets of interest downstream of the DISC

Although we show in chapter 3 and 6 that FLIP inhibition enhances apoptosis induction in colon cancer cell lines, it may not be sufficient in these type II cell lines that strongly depend on the mitochondrial amplification loop for caspase 3 activation (11). Our results in chapter 6 suggest that combining drug-induced inhibition of FLIP with drug-induced inhibition of the anti-apoptotic mitochondrial protein Mcl-1 strongly enhances TRAIL sensitivity in colon cancer cells. Others have shown that a double-hit strategy, including inhibition of both FLIP and XIAP expression, can stimulate TRAIL-induced apoptosis both *in vitro* and *in vivo* in a xenograft model (11). This might open the way for combinations that include standard chemotherapy and TRAIL receptor-targeting agents, supplemented by (specific) agents inactivating key inhibitors of the TRAIL apoptotic pathway. Targeting key blockers could be either tumor (sub) type-specific or patient-tailored. Three critical inhibition levels, namely the DISC (via FLIP), the mitochondria (via Bcl-2, Bcl-XL, Mcl-1) or the inhibitor of apoptosis proteins (via XIAP, cIAP1, cIAP2, survivin) offer interesting pools of potential therapeutic targets in colorectal cancer. Data show that some anti-apoptotic proteins such as the Bcl-2 family member Mcl-1 (12) are further upregulated in cancer cells after stimulation of the TRAIL apoptotic pathway, via a negative feedback loop preventing TRAIL-induced apoptosis. Similarly, tumor necrosis factor- α (TNF- α) induces activation of the NF κ B pathway. The subsequent FLIP upregulation acts as a potent switch that determines whether a pro-apoptotic or pro-survival effects will be induced by TNF- α . (13,14). Future research should determine which of the key proteins discovered *in vitro* are most relevant for TRAIL sensitivity *in vivo*, either in a broad context or in a tumor-specific perspective, for instance based on immunohistochemical studies. Basal protein levels, in absence of stimulation of the TRAIL receptor(s), may not be sufficient to predict sensitivity to

TRAIL receptor ligands. These studies should therefore consider the importance of the anti-apoptotic proteins that play a critical role in TRAIL sensitivity but are only detectable after stimulation of the TRAIL receptors.

MiRNA and TRAIL receptor-mediated apoptosis

First discovered in 1993, microRNAs (miRNAs) are small non-coding RNAs (19-25 nucleotides-long) that can block mRNA transcription and/or negatively regulate mRNA stability. MiRNAs possess oncogenic and tumor suppressing activities. As a consequence, the expression of these miRNAs is often deregulated in cancer. The oncogene MET upregulates miR-221&222 in aggressive non-small cell lung cancers and hepatocellular carcinomas. By inhibiting PTEN and TIMP3 expression, miR-221&222 induced TRAIL resistance (15). MiR29b, a negative regulator of Mcl-1 expression, was found to be downregulated in malignant cholangiocarcinomas, thereby preventing TRAIL-induced apoptosis (16). Interestingly, miR29b is also downregulated in colorectal cancer cells compared to normal (17). It is also worth noting that some DISC proteins are putative miRNA targets (18,19). Future research should determine whether one or several of these miRNA constitute good marker(s) for TRAIL sensitivity, which may also be improved by therapeutic targeting of the anti-apoptotic miRNAs.

Dual-drug combinations as a strategy to increase sensitivity to TRAIL death receptor-mediated apoptosis

Various drugs can inhibit the expression of anti-apoptotic proteins and/or upregulate pro-apoptotic proteins. So far, most studies have focused on combining single agents with TRAIL receptor-targeted therapies. In chapter 6, we show that combinations of two different drugs may achieve TRAIL-sensitizing effects far superior to the effects obtained from combining TRAIL with two drugs separately. Sorafenib and aspirin effectively increased TRAIL sensitivity in a panel of colon cancer cell lines, via combined inhibition of FLIP and Mcl-1. In medulloblastoma and neuroblastoma cells, combining a demethylating agent or histone deacetylase inhibitors with IFN- γ induced potent caspase 8 upregulation. Consequently, treatment with a combination of these drugs increased TRAIL sensitivity both *in vitro* and *in vivo* in a chorioallantoic membrane model (20,21). These data further support the rationale for similar dual-drug combinations in colorectal cancer. Since 5-FU constitutes an important part of adjuvant

and palliative treatments for colorectal cancer, integrating the interactions of 5-FU with other treatment options might be critical for improving TRAIL sensitivity in colon cancer cells. 5-FU increase TRAIL-sensitivity in colon cancer cells (22). However, 5-FU also stimulates the NF- κ B pathway, therefore hampering TRAIL-induced apoptosis (23). Similarly, 5-FU stimulates Mcl-1 mRNA levels and protein expression in pancreatic cancer cells with acquired resistance to this chemotherapeutic agent (24). Thus, inhibitors of the NF- κ B pathway or of Mcl-1 might increase the pro-apoptotic effects of 5-FU on TRAIL-induced apoptosis in cancer cells. Altogether, TRAIL receptor-targeting therapies should benefit from a better knowledge on drug-drug interactions.

TRAIL and colorectal cancer chemoprevention

Because of the slow progression of the adenoma-carcinoma sequence, colorectal cancer is particularly suitable for chemoprevention, notably on patients genetically predisposed to this disease. Although NSAIDs have shown some activity, truly effective chemopreventive drugs for colorectal cancer are lacking (25). Here, we have demonstrated that the expression of Mcl-1 is significantly increased in adenomas, including those of patients with Lynch syndrome. It remains to be determined whether the high Mcl-1 expression contributes to adenoma formation and development, for instance by blocking apoptosis. Early changes in expression of proteins belonging to the Bcl-2 family have been found during colorectal carcinogenesis (26). ABT-263, Genasense, TW-37, HA14-1 and Obatoclax are novel inhibitors specific for some or several proteins of the Bcl-2 family (27). These agents, currently in clinical trials, might help shifting the balance toward apoptosis. TRAIL receptor-agonists are generally well-tolerated in cancer patients and rhTRAIL induce apoptosis in colorectal adenoma cells (28). Combining NSAIDs with TRAIL receptor agonists or Bcl-2 family inhibitors might further increase the efficacy of these agents in chemopreventive settings.

Conclusions

Finally, early clinical studies performed in extensively pre-treated patients did not allow to determine whether TRAIL is the natural born killer of cancer cells that many had hoped it would be based on preclinical work. The study of the TRAIL apoptotic pathway has, however, already increased our mechanistic understanding of apoptosis

and its regulation in cancer cells. Novel key proteins have been defined, targeting of which could be beneficial to both TRAIL receptor-mediated apoptosis and chemotherapy-induced apoptosis. As demonstrated in this thesis, efficient DISC activity, and more particularly a favorable (high) caspase 8/FLIP ratio, is critical for efficient stimulation of the TRAIL apoptotic pathway *in vitro*. Enhancing caspase 8 cleavage at the DISC might also hold the key to TRAIL death receptor-mediated apoptosis *in vivo*. Rational application in the clinic of the knowledge obtained in preclinical models will increase the potential to translate TRAIL death receptor-mediated apoptosis into therapies that improve the prognosis of patients.

References

1. Hector S and Prehn J. Apoptosis signaling proteins as prognostic biomarkers in colorectal cancer: A review. *BBA-Rev Cancer* 2009; 1795: 117-129.
2. Pennarun B, Meijer A, de Vries EGE, Kleibeuker JH, Kruyt FA and de Jong S. Playing the DISC: Turning on TRAIL death receptor-mediated apoptosis in cancer. *BBA-Rev Cancer* 2010; 1805: 123-140.
3. van Geelen CMM, de Vries EGE and de Jong S. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist Updat* 2004; 7: 345-358.
4. Mahalingam D, Szegezdi E, Keane M, de Jong S and Samali A. TRAIL receptor signalling and modulation: Are we on the right TRAIL? *Cancer Treat Rev* 2009; 35: 280-288.
5. Falschlehner C, Emmerich CH, Gerlach B and Walczak H. TRAIL signalling: decisions between life and death. *Int J Biochem Cell Biol* 2007; 39: 1462-1475.
6. Marini P, Denzinger S, Schiller D, Kauder S, Welz S, Humphreys R, Daniel PT, Jendrossek V, Budach W and Belka C. Combined treatment of colorectal tumours with agonistic TRAIL receptor antibodies HGS-ETR1 and HGS-ETR2 and radiotherapy: enhanced effects in vitro and dose-dependent growth delay in vivo. *Oncogene* 2006; 25: 5145-5154.
7. Ndozangue-Touriguine O, Sebbagh M, Merino D, Micheau O, Bertoglio J and Breard J. A mitochondrial block and expression of XIAP lead to resistance to TRAIL-induced apoptosis during progression to metastasis of a colon carcinoma. *Oncogene* 2008; 27: 6012-6022.
8. Vigneswaran N, Wu J, Nagaraj N, Adler-Storthz K and Zacharias W. Differential susceptibility of metastatic and primary oral cancer cells to TRAIL-induced apoptosis. *Int J Oncol* 2005; 26: 103-112.
9. Longley DB, Wilson TR, McEwan M, Allen WL, McDermott U, Galligan L and Johnston PG. c-FLIP inhibits chemotherapy-induced colorectal cancer cell death. *Oncogene* 2006; 25: 838-848.
10. Wilson TR, McLaughlin KM, McEwan M, Sakai H, Rogers KM, Redmond KM, Johnston PG and Longley DB. c-FLIP: a key regulator of colorectal cancer cell death. *Cancer Res* 2007; 67: 5754-5762.
11. Wilson TR, McEwan M, McLaughlin K, Le Clorennec C, Allen WL, Fennell DA, Johnston PG and Longley DB. Combined inhibition of FLIP and XIAP induces Bax-independent apoptosis in type II colorectal cancer cells. *Oncogene* 2009; 28: 63-72.
12. Ricci MS, Kim SH, Ogi K, Plastaras JP, Ling J, Wang W, Jin Z, Liu YY, Dicker DT, Chiao PJ, Flaherty KT, Smith CD and El-Deiry WS. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* 2007; 12: 66-80.
13. Van Antwerp DJ, Martin SJ, Kafri T, Green DR and Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science* 1996; 274: 787-789.
14. Wang L, Du F and Wang X. TNF-alpha induces two distinct caspase-8 activation pathways. *Cell* 2008; 133: 693-703.
15. Garofalo M, Di Leva G, Romano G, Nuovo G, Suh SS, Ngankeu A, Taccioli C, Pichiorri F, Alder H, Secchiero P, Gasparini P, Gonelli A, Costinean S, Acunzo M, Condorelli G and Croce CM. miR-

- 221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. *Cancer Cell* 2009; 16: 498-509.
16. Mott JL, Kobayashi S, Bronk SF and Gores GJ. mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* 2007; 26: 6133-6140.
17. Cummins JM, He Y, Leary RJ, Pagliarini R, Diaz LA, Jr., Sjoblom T, Barad O, Bentwich Z, Szafranska AE, Labourier E, Raymond CK, Roberts BS, Juhl H, Kinzler KW, Vogelstein B and Velculescu VE. The colorectal microRNAome. *Proc Natl Acad Sci U S A* 2006; 103: 3687-3692.
18. Garofalo M, Quintavalle C, Di LG, Zanca C, Romano G, Taccioli C, Liu CG, Croce CM and Condorelli G. MicroRNA signatures of TRAIL resistance in human non-small cell lung cancer. *Oncogene* 2008; 27: 3845-3855.
19. Ovcharenko D, Kelnar K, Johnson C, Leng N and Brown D. Genome-scale microRNA and small interfering RNA screens identify small RNA modulators of TRAIL-induced apoptosis pathway. *Cancer Res* 2007; 67: 10782-10788.
20. Fulda S and Debatin KM. 5-Aza-2'-deoxycytidine and IFN-gamma cooperate to sensitize for TRAIL-induced apoptosis by upregulating caspase-8. *Oncogene* 2006; 25: 5125-5133.
21. Hacker S, Dittrich A, Mohr A, Schweitzer T, Rutkowski S, Krauss J, Debatin KM and Fulda S. Histone deacetylase inhibitors cooperate with IFN-gamma to restore caspase-8 expression and overcome TRAIL resistance in cancers with silencing of caspase-8. *Oncogene* 2009.
22. Lacour S, Micheau O, Hammann A, Drouineaud V, Tschopp J, Solary E and manche-Boitrel MT. Chemotherapy enhances TNF-related apoptosis-inducing ligand DISC assembly in HT29 human colon cancer cells. *Oncogene* 2003; 22: 1807-1816.
23. Voboril R, Hochwald S, Li J, Brank A, Weberova J, Wessels F, Moldawer L, Ramsay Camp E and MacKay S. Inhibition of NF-Kappa B augments sensitivity to 5-Fluorouracil/Folinic acid in colon cancer1. *J Surg Res* 2004; 120: 178-188.
24. Shi X, Liu S, Kleeff J, Friess H and Buchler MW. Acquired resistance of pancreatic cancer cells towards 5-Fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. *Oncol* 2002; 62: 354-362.
25. Jalving M, Koornstra JJ, de Jong S, de Vries EGE and Kleibeuker JH. Review article: the potential of combinational regimen with non-steroidal anti-inflammatory drugs in the chemoprevention of colorectal cancer. *Aliment Pharmacol Ther* 2005; 21: 321-339.
26. Koornstra JJ, Jalving M, Rijcken F, Westra J, Zwart N, Hollema H, de Vries EGE, Hofstra R, Plukker J, Jong S and Kleibeuker JH. Expression of tumour necrosis factor-related apoptosis-inducing ligand death receptors in sporadic and hereditary colorectal tumours: Potential targets for apoptosis induction. *Eur J Cancer* 2005; 41: 1195-1202.
27. Chonghaile TN and Letai A. Mimicking the BH3 domain to kill cancer cells. *Oncogene* 0 AD; 27: S149-S157.

28. Jalving M, de Jong S, Koornstra JJ, Boersma-van Ek W, Zwart N, Wesseling J, de Vries EGE and Kleibeuker JH. TRAIL induces apoptosis in human colorectal adenoma cell lines and human colorectal adenomas. Clin Cancer Res 2006; 12: 4350-4356.

Chapter 8

Summary in Dutch (Nederlandse samenvatting)

Samenvatting van de resultaten

Darmkanker is de op één na grootste veroorzaker van kanker-gerelateerde doodsoorzaken in westerse landen. Hoewel er therapeutisch gezien vooruitgang geboekt is, blijft intrinsieke en/of verkregen resistentie tegen cytostatica een probleem. Hoogstwaarschijnlijk wordt deze resistentie veroorzaakt door verstoring van de route van geprogrammeerde celdood, oftewel apoptose, in darmkankercellen.

In de literatuur worden anti-tumor en anti-metastasering eigenschappen toegedicht aan de tumor necrosis factor-gerelateerde apoptose-inducerende ligand (TRAIL) apoptoseroute. Dit maakt deze route een aantrekkelijk therapeutisch doel voor antikankerbehandeling. Er zijn nieuwe medicijnen ontwikkeld - waaronder recombinant humane TRAIL en agonistische antilichamen, die ofwel TRAIL receptor 1 of TRAIL receptor 2 (TRAIL-R1 of -R2) kunnen aanzetten om de extrinsieke apoptoseroute in kankercellen te stimuleren. Combinatietherapieën die deze middelen gebruiken worden momenteel geëvalueerd op hun vermogen om apoptose in cytostatica-resistente cellen te herstellen. In het onderzoek, zoals beschreven in dit proefschrift, worden verschillende aspecten van de TRAIL apoptoseroute bekeken, met als doel het aanpakken van moleculaire doelwitten van deze route in darmkanker te verbeteren.

Ten eerste wordt in **hoofdstuk 2** de huidige kennis betreffende de eerste stap in deze route, het zogenaamde death inducing signaling complex (DISC) oftewel het celdoodinducerende signaleringscomplex beschreven, om zo nieuwe strategieën te definiëren, die tot een hogere gevoeligheid voor TRAIL death receptor-gemedieerde apoptose zouden kunnen leiden.

De samenstelling van de DISC wordt in kankercellen geïnitieerd via het binden van TRAIL aan een van zijn proapoptotische death receptoren, TRAIL-R1 of TRAIL-R2. Componenten van de DISC zijn trimeren TRAIL-R1/R2 in complex met FADD, caspase 8 en caspase 10. Wanneer het anti-apoptotische eiwit FLIP naast caspase 8 deel uitmaakt van de DISC, wordt een inactief complex gevormd. De activering van caspase 8/10 in een DISC initieert de caspasecascade, die uiteindelijk leidt tot geprogrammeerde celdood. Er zijn naast TRAIL en TRAIL-R1- en TRAIL-R2-selectieve varianten van TRAIL ook agonistische antilichamen ontwikkeld, die specifiek gericht zijn tegen TRAIL-R1 of TRAIL-R2. Deze liganden zijn interessant als antikanker medicijnen,

aangezien ze selectief tumorcellen doden. Om de gevoeligheid voor TRAIL death receptor-gemedieerde apoptose in de tumor te verhogen en om cytostaticaresistentie te omzeilen, zijn in preklinische modellen al TRAIL receptorliganden gecombineerd met verschillende andere medicamenten. In **hoofdstuk 2** worden factoren die de eerste stappen in de TRAIL apoptosesignaalroute beïnvloeden besproken, waarbij de focus gelegd wordt op mechanismen die de DISC vorming kunnen moduleren, en op het activeren van caspases in de DISC. Deze inzichten kunnen zo het ontwerpen van verdere studies sturen, waarin medicijnen worden gecombineerd met TRAIL receptorliganden om zo een maximale DISC signalering te krijgen.

Een manier, om combinaties die TRAIL-geïnduceerde apoptose verhogen te identificeren, is het ontwikkelen van kankercellijnen die resistent gemaakt zijn voor TRAIL. Hierbij zijn in het verleden vaak darmkankercellen, die al een verminderde capaciteit voor reparatie van DNA mismatches hebben, gebruikt. Deze kankercellen hebben vaak mutaties in genen die tandem repeats in het DNA bevatten, waaronder het gen voor het pro-apoptotische eiwit Bax. Tumoren met deze eigenschappen vertegenwoordigen echter slechts een klein gedeelte van de darmkankers (15-20%). In **hoofdstuk 3** onderzochten we het onderliggende mechanisme voor het verkrijgen van resistentie tegen TRAIL in een darmkankercellijn, die nog wel een intact mismatch-herstel-mechanisme bevatte. De TRAIL-resistente cellijn SW948-TR werd verkregen uit de TRAIL-gevoelige cellijn SW948 door constante blootstelling van de cellen aan rhTRAIL. De SW948-TR cellen vertonen een 140 keer verlaagde gevoeligheid voor TRAIL in een overlevingsassay. In afwezigheid van TRAIL bleven de cellen resistent gedurende meer dan één jaar. Beide cellijnen vertoonden een vergelijkbaar TRAIL death receptor-expressieniveau op de celmembraan. Behandeling van de cellen met de eiwitsyntheseremmer cycloheximide maakte de SW948-TR cellen gevoelig voor TRAIL-geïnduceerde apoptose, wat een aanwijzing was dat de TRAIL death receptoren niet gemuteerd waren, maar dat de resistentie op een ander niveau in de cel zit.

De caspase 8 eiwitspiegels in SW948-TR cellen waren duidelijk lager dan in SW948 cellen. Caspase 8 mRNA niveaus in de twee cellijnen waren niet verschillend. Downregulatie van c-FLIP met short interfering RNA (siRNA) verhoogde de gevoeligheid van SW948-TR cellen voor TRAIL, terwijl caspase 8 siRNA de TRAIL gevoeligheid in SW948 cellen verlaagde. Dit onderstreept het belang van de caspase 8/FLIP ratio. Proteasoomremming met MG-132 zorgde niet voor een herstel van het

caspase 8 basisniveau in SW948-TR cellen, maar stabiliseerde het door TRAIL geactiveerde caspase 8 in deze cellen. Onze resultaten suggereren dat darmkankercellen TRAIL resistent worden door een verlaging van de caspase 8/FLIP ratio en door een verhoogde afbraak van actief caspase 8 na TRAIL behandeling. Proteasoomremmers zouden deze verkregen TRAIL resistentie kunnen terugdraaien in met name darmkankercellen die een intact mismatch-repair-systeem bevatten.

Er zijn verschillende medicijnen ontwikkeld die op de TRAIL death receptoren aangrijpen, zoals TRAIL en TRAIL-R1/R2-specifieke antilichamen. Omdat verschillende liganden ook verschillende eigenschappen kunnen hebben, onderzochten we in **hoofdstuk 4** de bijdrage van TRAIL-R1 en TRAIL-R2 aan de TRAIL-gevoeligheid en -resistentie in SW948 en SW948-TR cellen. Ook bepaalden we of TRAIL-R1 of TRAIL-R2 agonistische antilichamen de TRAIL resistentie zouden kunnen opheffen. SW948 cellen waren gevoelig voor alle drie de TRAIL death receptor-liganden, hoewel het TRAIL-R2 antilichaam slechts zwakke caspase 8 activering en lage apoptose liet zien. Toch induceerden de anti-TRAIL-R1 en anti-TRAIL-R2 antilichamen gelijkwaardige DISC vorming en was er ongeveer evenveel geactiveerd caspase 8 in de DISC aanwezig in SW948. Dit is een aanwijzing dat verdere caspase 8 activering geremd wordt na stimulering van TRAIL-R2. SW948-TR cellen waren resistent voor alle TRAIL death receptor-liganden door de lagere caspase 8 eiwitniveaus en dus minder caspase 8 activering. Caspase 8 niveaus konden worden hersteld in SW948-TR door voorbehandeling met MG-132 of interferon-gamma (IFN- γ), waarna eveneens de gevoeligheid voor rhTRAIL en TRAIL-R1 antilichaam verhoogd werd. Verrassend genoeg vergrootte MG-132 ook TRAIL-R2 gemedieerde apoptose. Deze resultaten benadrukken dat er een kritiek verschil bestaat tussen TRAIL-R1 en TRAIL-R2 apoptotische signalering, hetgeen mogelijk gevolgen heeft voor toekomstige combinaties met TRAIL death receptor-liganden.

De activiteit van de insulin-like growth factor 1 (IGF-1) “pro-survival” route is vaak verhoogd in kankercellen, hetgeen invloed heeft op celdelingsactiviteit en apoptose. In **hoofdstuk 5** stellen we dat remming van de IGF-1 receptor (IGF-1R) de TRAIL death receptor-gemedieerde apoptose zal verhogen in darmkankercellen. NVP-AEW541, een IGF-1R tyrosinekinaseremmer met laag moleculair gewicht, verlaagde de gevoeligheid voor TRAIL en voor een TRAIL-R1 antilichaam, maar verrassend genoeg werd de gevoeligheid voor een TRAIL-R2 antilichaam verhoogd. NVP-AEW541 remde

de IGF-1 geïnduceerde activering van phosphatidylinositol 3-kinase (PI3K) route. De effecten van de PI3K remmer LY294002 op TRAIL-geïnduceerde apoptose waren vergelijkbaar met die van NVP-AEW541, wat de rol voor IGF-1R gemedieerde activering van PI3K onderbouwt. We hebben laten zien dat PI3K remming de TRAIL-R2 gemedieerde caspase 8 activering verhoogde, maar tegelijkertijd de TRAIL-R1 membraanexpressie en TRAIL-R1-gemedieerde caspase 8 activering verlaagde. Daarnaast toonden we aan dat remming van PI3K de TRAIL gevoeligheid verlaagde. Dit effect is onafhankelijk is van de initiële voorkeur van de cellijn voor TRAIL-R1 of TRAIL-R2 gemedieerde apoptosesignalering. Deze resultaten suggereren dat rekening gehouden moet worden met individuele effecten op TRAIL-R1 of TRAIL-R2 gemedieerde apoptosesignalering, wanneer TRAIL receptorliganden gecombineerd worden met PI3K remmers.

De multikinaseremmer sorafenib is in de kliniek effectief bij bepaalde kankertypes, en voorkomt celdeling van darmkankercellen *in vitro*. Niet-steroïde anti-inflammatoire medicijnen zoals acetylsalicylzuur (aspirine) remmen darmkankercellen *in vitro*. Het doel in **hoofdstuk 6** was om te bepalen of de combinatie van aspirine met sorafenib de celdelingremmende effecten van beide medicijnen versterkt, en of de combinatie tevens de TRAIL-geïnduceerde apoptose in menselijke darmkankercellijnen (SW948, Lovo, Colo205, Colo320, Caco-2 en HCT116) vergroot. Aspirine versterkte de celdelingremmende werking van sorafenib in vier cellijnen zeer sterk (ongeveer 4 keer verhoogd) door het remmen van de celcyclus.

De combinatie van lage doseringen van aspirine ($\leq 2,5$ mM) en sorafenib ($\leq 2,5$ μ M) verhoogde de gevoeligheid van TRAIL-gevoelige en -resistente darmkankercellen voor TRAIL sterk. De oorzaak voor de toegenomen TRAIL gevoeligheid was een verlaging van de cellulaire spiegels van de anti-apoptotische eiwitten FLIP en Mcl-1 na combinatiebehandeling met aspirine en sorafenib, wat werd bevestigd met specifieke verlaging van deze eiwitten met behulp van small interfering RNA.

Vervolgens werd de klinische relevantie van het aangrijpen op FLIP en Mcl-1 in darmkanker onderzocht. Immunohistochemie op weefsel verkregen van patiënten wees uit dat Mcl-1 expressie significant verhoogd was in darm-adenomen en -carcinomen vergeleken met gezond darmepitheel, wat vergelijkbaar was met de verhoogde FLIP expressiewaarden die recentelijk gezien werden in darmkankerweefsel met behulp van immunohistochemie. Deze resultaten benadrukken het belang van het combineren van

lage doseringen aspirine en sorafenib om de celdeling te remmen en de expressie van de anti-apoptotische eiwitten FLIP en Mcl-1 te verlagen. Verder pre(klinisch) onderzoek zal moeten uitwijzen wat de effecten van deze medicijncombinatie met TRAIL receptorliganden zijn bij darmkanker.

Conclusies

De veelbelovende resultaten met TRAIL receptorliganden in preklinisch onderzoek hebben geleid tot klinische studies. De eerste klinische studies uitgevoerd in patiënten die al vele voorafgaande behandelingen hadden ondergaan, maken het echter nog niet mogelijk om iets te zeggen over de bijdrage van TRAIL receptorliganden in de behandeling van darmkankerpatienten. Het onderzoek aan de TRAIL apoptoseroute heeft ons al wel veel geleerd heeft over apoptose en de regulatie van apoptose in kankercellen. Nieuwe eiwitten, die een centrale rol spelen in apoptose, zijn geïdentificeerd. Het richten van medicijnen op deze eiwitten kan een voordeel zijn om zo samen met TRAIL receptor liganden of in combinatie met cytostatica meer apoptose te krijgen in darmkankercellen. Zoals aangetoond in *in vitro* modellen in dit proefschrift, is een effectieve DISC formatie en activering van caspase 8 die vooral afhangt van een hoge caspase 8/FLIP ratio essentieel om apoptose via de TRAIL receptorenroute te induceren. Efficiëntere activering van caspase 8 in de DISC door specifiek op TRAIL-R1 of TRAIL-R2 gerichte liganden te gebruiken in combinatie met caspase 8 expressie verhogende medicijnen zou een mogelijke oplossing zijn om ook *in vivo* TRAIL death receptor gemedieerde apoptose te krijgen. Rationele toepassing in de kliniek van de kennis verkregen uit preklinische modellen zou dan mogelijk kunnen leiden tot op TRAIL receptor gerichte therapieën, die daadwerkelijk de prognose van darmkanker patiënten kunnen verbeteren.

Acknowledgements

As my Ph.D. is now drawing to a close, I finally have the chance to take a minute and look back. A Ph.D. is definitely a long journey but not a lonely one. I am glad to be able to properly acknowledge here all the people who have shared my work and life throughout these years spent in Groningen.

First of all, my first promotor Prof.dr. Jan Kleibeuker, thank you for taking me on as a Ph.D. student. No one better than a gastroenterologist could have provided me, as a biologist working on colon cancer, with the appropriate clinical feedback on my research. Your sense of detail has been really helpful for spotting critical mistakes that most would miss in a manuscript, and for helping make the confusing sections of a manuscript intelligible. I would like to thank my second promotor, Prof.dr. Liesbeth de Vries, for initially putting me in contact with Steven de Jong, and for her involvement all along the way. Liesbeth, your hard work and determination are legendary. Among other things, this means for your Ph.D. students both timely feedback on manuscripts and the incentive to meet deadlines. Being able to count on you for these things has meant a lot to me. I have learned a lot from you on how to keep in mind the translational aspects of research. My supervisor, Dr. Steven de Jong, sharing research ideas with you has been a real pleasure. I hope you have enjoyed our brainstorming sessions as much as I have. There are no boundaries to your scientific imagination. I greatly appreciate your encouragement to express and pursue my own ideas, ideas you have also taught me to defend. Thanks again for your inspiration. Dr. Frank Kruijt, thanks for your valuable feedback during meetings and for initiating many stimulating discussions. Prof.dr. Harry Hollema, I am grateful that you found the time to look at our IHC slides in spite of your busy schedule. Thanks to Dr. Steffan Nawrocki for performing the ongoing xenograft mouse study.

I would like to thank the members of the reading committee Prof.dr. Wim Quax, Prof.dr. Ate van der Zee and Prof.dr. Han Moshage for spending some of their valuable time evaluating my thesis.

Hetty and Coby, you are the “invisible” hands running the lab. I cannot imagine what the Ph.D. experience would be like if students had to take care themselves of all the administrative tasks you discreetly accomplish for them. I admire your ability to react

calmly to the constant flow of people who walk in your room with questions while you are trying to work on the computer. Special thanks to Hetty for her help filling out indecipherable forms and to Coby for the many emergency orders she didn't mind arranging. Thanks also to Gretha and Bianca for their help with my administrative requests.

Phuong and Wytke, thanks for your help in the lab, especially during the last part of the Ph.D. when I didn't have enough hands to do it all. Annemieke, the review we wrote together was definitely worth the time spent behind the computer trying to figure out how to properly merge reference databases. I wish you good luck with your own thesis. Thanks to all the Ph.D. students, and in particular to those who formed the core of the apoptosis group (including Roelof, Janet, Dianne, Shinta). I am also very grateful to all the technicians for their patient input with lab-work related questions (naïve ones included). In particular I want to thank Gert Jan, Haukeline, Mirjam and Dorenda, who were (almost) there all along my Ph.D. Thanks also to Eli for his critical involvement in the experiments with radio-labeled TRAIL. Thanks to Deva and Carlos for the very stimulating discussions.

Good luck to all the new Ph.D. students who have started in the last year or so. It's nice to see some fresh blood coming into the group, and it reminds me (in a good way) of myself a few years ago. Tjitske, I am glad you are now working on your own Ph.D. after almost a year spent working on TRAIL with me during one of your Master's projects. Thanks also to all my office mates Xiang-Yi, Yang Nan, Sarah, Linda, Esther, Arne, Hein and Lina for the constant alternation of fun and studiousness. Esther, I am glad you could translate my thesis summary into Dutch. Arne, no one can entertain an office better than you. Thanks to the changeable group of late lunchers (including Esther, Agnes, Millind and Justin, who weren't mentioned above). You made eating at irregular hours much more enjoyable.

Outside the lab, many thanks to the group of "real biologists" for the many holiday-themed dinners, break-dance battles as well no-dancing parties, and the weekends away from Groningen in Amsterdam and Germany. I never made it once to the Monday beer event, but Groningen wouldn't have been the same without you.

Special thanks to my friend Sami for agreeing to come back to Groningen and be my paranymp. Sami, your genuine enthusiasm and hyper-activity, both in science and everyday life, has been highly inspiring.

Special thoughts go to my family for their love and patience. Spending all this time away from them has been as challenging in itself as doing a Ph.D., but I am glad for their unconditional support.

Finally, my heart goes to my dear Kristin for being both my love and my family away from home. Kristin, thanks for your support and inspiration to do better.

May 15, 2010

Bodvaël

